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Immunosuppressant PG490 (Triptolide) Inhibits T-cell Interleukin-2 Expression at the Level of Purine-box/Nuclear Factor of Activated T-cells and NF-κB Transcriptional Activation*

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Daoming Qiu, Guohua Zhao, Yosuke Aoki, Lingfang Shi, Anne Uyei, Saman Nazarian, James C.-H. Ng, and Peter N. Kao‡

From the Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, California 94305-5236

PG490 (triptolide) is a diterpene triepoxide with potent immunosuppressive and antiinflammatory properties. PG490 inhibits interleukin(IL)-2 expression by normal human peripheral blood lymphocytes stimulated with phorbol 12-myristate 13-acetate (PMA) and antibody to CD3 (IC_{50} of 10 ng/ml), and with PMA and ionomycin (Iono, IC_{50} of 40 ng/ml). In Jurkat T-cells, PG490 inhibits PMA/Iono-stimulated IL-2 transcription. PG490 inhibits the induction of DNA binding activity at the purine-box/antigen receptor response element (ARRE)/nuclear factor of activated T-cells (NF-AT) target sequence but not at the NF-κB site. PG490 can completely inhibit transcriptional activation at the purine-box/ARRE/NF-AT and NF-κB target DNA sequences triggered by all stimuli examined (PMA, PMA/Iono, tumor necrosis factor-α). PG490 also inhibits PMA-stimulated activation of a chimeric transcription factor in which the C-terminal TA1 transactivation domain of NF-κB p65 is fused to the DNA binding domain of GAL4. In 16HBE human bronchial epithelial cells, IL-8 expression is regulated predominantly by NF-κB, and PG490 but not cyclosporin A can completely inhibit expression of IL-8. The mechanism of PG490 inhibition of cytokine gene expression differs from cyclosporin A and involves nuclear inhibition of transcriptional activation of NF-κB and the purine-box regulator operating at the ARRE/NF-AT site at a step after specific DNA binding.

Extracts of the Chinese herb Tripterygium Wilfordii hook have potent antiinflammatory and immunosuppressive properties and have been used successfully in traditional Chinese medicine for the treatment of rheumatoid arthritis (1). One active component of Tripterygium extracts is the diterpene triepoxide, triptolide, which possesses antileukemic activities (2) and also inhibits proliferation of transformed cell lines (3, 4). Kupchan and Shubert (5) described that triptolide possesses a 9,11-epoxy-14β-hydroxy system, which is important for biologic activity, and proposed that this system may be involved in selective alkylation by nucleophilic groups such as thiols present in key target enzymes involved in growth regulation.

A refined extract of Tripterygium, PG27, which contains PG490 (triptolide) as its active compound, prolongs heart and kidney allograft survival in rat transplantation models and, furthermore, displays synergy with the immunosuppressant cyclosporin A (CsA)[1] in preventing cardiac and renal allotransplant rejection.[2] The combination of PG27 with CsA substantially prolongs hamster cardiac xenograft survival in rat recipients and inhibits the production of serum anti-hamster IgM and IgG xenoantibodies where single drug therapies are ineffective.[2] In addition, PG27 suppresses the development of graft *versus* host disease associated with allogeneic bone marrow transplantation.[3] The chloroform methanol extract of Triptyergium, T2 (6), has been studied recently (7–9) and was shown to block mitogen-induced early cytokine gene transcription in T-cells (10).

An early cytokine transcribed during T-cell activation is IL-2 (reviewed in Ref. 11). IL-2 transcription involves specific DNA binding and transcriptional activation of a purine-box transcriptional regulator operative at the antigen receptor response element (ARRE)/NF-AT target DNA sequence, and of NF-κB, AP-1, and Oct-1 (reviewed in Ref. 11). The T-cell immunosuppressants, CsA and FK506, inhibit transcription of the IL-2 gene through mechanisms that may involve the serine/threonine protein phosphatase, calcineurin (reviewed in Ref. 11).

Cyclosporin A and FK506 interfere with the induction of sequence-specific DNA binding activity at the purine-box/ARRE/NF-AT target DNA sequence (reviewed in Ref. 11). We previously purified to homogeneity a CsA- and FK506-sensitive sequence-specific purine-box DNA binding complex that contains NF45 and NF90 proteins (12, 13). Recently, we showed that NF45 and NF90 associate tightly with the catalytic subunit of DNA-dependent protein kinase and serve to stabilize the association of the catalytic subunit of DNA-dependent protein kinase with DNA-targeting proteins, Ku80 and Ku70 (14). The catalytic subunit of DNA-dependent protein kinase and Ku have been shown to bind with sequence specificity to purine-rich target DNA sequences and to mediate sequence-specific transcriptional repression (15). We have recently shown that the specific CsA-sensitive purine-box/ARRE DNA binding complex in human bronchial epithelial cells involves NF45, NF90, Ku80, and Ku70 with no evidence for NF-ATp or NF-ATc proteins (16).

Activation of IL-2 transcription triggered through costimu-

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‡ To whom correspondence should be addressed. Tel.: 650-725-0570; Fax: 650-725-5489; E-mail: peterkao@leland.stanford.edu.

[1] The abbreviations used are: CsA, cyclosporin A; ARRE, antigen receptor response element; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde phosphate dehydrogenase; IL, interleukin; Iono, ionomycin; NF-AT, nuclear factor of activated T-cells; TNF, tumor necrosis factor; PG490, Pharmagenesis 490, 97% pure triptolide; PMA, phorbol 12-myristate 13-acetate; PBL, peripheral blood lymphocytes; bp, base pair(s); RLU, relative light units; IFN, interferon; P/I, PMA + ionomycin; ELISA, enzyme-linked immunosorbent assay; NS, nonstimulated.

[2] J. Fidler, Pharmagenesis, Palo Alto, CA, unpublished data.

[3] N. Chao, Duke University, and J. Fidler, Pharmagenesis, Palo Alto, CA, unpublished data.

latory receptors such as CD28 involves NF-κB (17) and is largely resistant to inhibition by CsA (17, 18). The activation of transcription by NF-κB involves stimulation-induced degradation of IκB, which serves to release NF-κB p65 for translocation from the cytoplasm into the nucleus. In the nucleus, p65 binds as a heterodimer to its target DNA sequence and then interacts with transcriptional regulatory components to signal initiation of transcription by RNA polymerase II (reviewed in Ref. 19). NF-κB signaling is regulated by phosphorylation in the cytoplasm at the level of IκB in the nucleus at the level of specific DNA binding and also at the level of transcriptional activation (reviewed in Ref. 20).

We investigated the immunosuppressive and antiinflammatory properties of PG490 (pure triptolide) in human peripheral blood lymphocytes, Jurkat T-cells, and human bronchial epithelial cells and show that the mechanism of inhibition by PG490 differs fundamentally from that of CsA and involves inhibition of transcriptional activation of the purine-box regulator of the ARRE/NF-AT site and of NF-κB at a step after specific binding to DNA.

EXPERIMENTAL PROCEDURES

Source of Triptolide—PG490 (triptolide, molecular weight 360) was obtained from Pharmagenesis (Palo Alto, CA). The material was composed of white to off-white crystals, had a melting point of 226–240 °C, conformed to standard triptolide preparation by proton nuclear magnetic resonance (2), and was 97% pure by reverse phase high pressure liquid chromatography evaluation using acetonitrile:methanol:water (18:9:73).[4]

Cell Culture and Stimulation Conditions—Human peripheral blood lymphocytes (PBL) were prepared by centrifugation on a gradient of sodium diatrizoate/Ficoll (Sigma) of a buffy coat obtained from the Stanford Hospital blood bank. Monocytes were depleted by adherence to plastic culture dishes for 30 min at room temperature and then the PBLs were stimulated for 12 h at a density of 1×10^7 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum. Jurkat T-cells (clone E6–1) were obtained from American Type Culture Collection (Manassas, VA), and cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (BioWhittaker, Walkersville, MD). An SV-40 large T-antigen transformed human bronchial epithelial cell line 16HBE14o- (16HBE), which retains differentiated morphology and function of normal human airway epithelia (21), was cultured in Eagle's minimum essential medium (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (BioWhittaker) as described (22). Human PBLs, Jurkat T-cells, or monolayer 16HBE cells were stimulated for the indicated times in culture media containing 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Calbiochem), PMA + antibody to CD3 (clone HIT3a, Pharmingen, San Diego, CA), PMA + antibody to CD28 (YTH913.12, BIOSOURCE, Camarillo, CA), PMA + 2 μM ionomycin (Iono; Calbiochem), or 20 ng/ml TNF-α (BIOSOURCE) in the presence of PG490, CsA (Sandoz), or FK506 (Fujisawa).

RNA Extraction and Northern Analyses—Total RNA was isolated and analyzed by Northern hybridization as described (22). Complementary DNA probes for human IL-2 (738 bp). IL-8 (289 bp), IκBα (883 bp), NF90 (2,008 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0.6-kilobase *Xba*I-*Hin*dIII fragment of cDNA) were labeled with [α-^{32}P]dCTP using a random hexamer labeling kit (Stratagene).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Jurkat T-cells were stimulated for 3 h, pelleted, and washed, and cytosolic and nuclear extracts were prepared as described (12). Briefly, nuclear proteins were extracted from chromatin using 0.3 M $(NH_4)_2SO_4$, and then soluble nuclear proteins were precipitated using 1.5 M $(NH_4)_2SO_4$, followed by dialysis into DNA binding buffer. Protein concentrations were determined by Bradford assay (Bio-Rad).

Transcription factor DNA binding activities in Jurkat T-cell nuclear extracts were assayed using electrophoretic mobility shift assays (EMSA). 10 μg of nuclear proteins were incubated for 30 min at 25 °C in 20 μl of binding buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 10% glycerol, 50 mM KCl, 0.05 mM dithiothreitol) containing 1–2 μg of poly(dI-dC) and 2.5 pg of ^{32}P-labeled oligonucleotide probe (approximately 1×10^5 cpm). The sequences of oligonucleotide probes used were agctAAAGAGGGACTTTCCCTAAA for the immunoglobulin κ light chain NF-κB site (19), and aagaAAGGAGGAAAAACTGTTTCATA (−259 to −284 in the human IL-2 enhancer) for the purine-box/NF-AT site (11). Probes were labeled by filling in overhanging ends (identified by lowercase letters) using Klenow DNA polymerase (New England Biolabs) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech), and nonradioactive dGTP, dATP, and dTTP. Protein-DNA complexes were resolved from free probe using 4% nondenaturing polyacrylamide gels in 0.5× Tris borate EDTA (pH 8.3) and visualized by fluorography.

Plasmids, Transfections, and Luciferase Reporter Gene Assays—Luciferase reporter gene constructs were under the control of the IL-2 enhancer (nucleotides −326 to +48, pCLN15deltaCX, prepared by D. Durand, Stanford University), or three copies of the purine-box/NF-AT regulatory sequence sequence (−285 to −255 of the human IL-2 enhancer) in the context of the minimal IL-2 promoter, or the immunoglobulin κ light chain NF-κB sequence monomer in the context of the minimal IL-8 promoter (−45 to +40 of the human IL-8 promoter). These plasmids also contain a neomycin resistance gene under control of the constitutively active SV40 promoter, and this allows G418 antibiotic (Life Technologies, Inc.) selection of cell lines that stably express the luciferase reporter constructs. The GAL4-luciferase reporter contains five copies of the GAL4 target DNA sequence upstream of the minimal IL-2 promoter and was prepared by J. Riegel at Stanford University. For normalizing the transient transfection assays, plasmid pEF Renilla luciferase was generated by cloning the elongation factor 1α promoter (23) into the pRL null vector (Promega) between the *Eco*RI and *Hin*dIII sites. Expression plasmids pEF CNA and pEF CNB contain the elongation factor 1α promoter (23) upstream of rat calcineurin A and calcineurin B cDNAs (24). Expression plasmids GAL4p65TA1 and GAL4p65{TA1+TA2} utilize the Rous sarcoma virus promoter to drive expression of a chimeric protein with the GAL4 DNA binding domain (amino acids 1–147) fused to the p65 transactivating domains and were generated by Schmitz and Baeuerle (25) and obtained through A. Baldwin (University of North Carolina, Chapel Hill).

Jurkat T-cells were transfected by electroporation with reporter and expression plasmids as described (13, 26). T-cells were stimulated for 6–12 h (24 h after electroporation in the case of transient transfections) and then cells were washed in phosphate-buffered saline and pelleted by centrifugation. Cell pellets were resuspended in 50 μl of lysis buffer (1% Triton X-100, 0.1 mM HEPES, pH 7.6, 1 mM dithiothreitol, and 2 mM EDTA, pH 8.0) for 10 min at 4 °C, then the cell lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were collected as whole cell extracts, and the Bradford reagent (Bio-Rad) was used to measure protein concentration. 20 μg of protein was mixed with 200 μl of luciferase reaction mixtures (1 mg/ml bovine serum albumin, 5 mM ATP, pH 7.6, 25 mM glycylglycine, and 15 mM $MgSO_4$) and 100 μl of 1 mM D-luciferin (Analytical Luminescence Laboratory, San Diego, CA). Triplicate determinations of luminescence were each read for 20 s using a Monolight™ 2010 luminometer (Analytical Luminescence Laboratory), and were measured in relative light units (RLU). In transient transfection experiments that incorporated the pEF Renilla luciferase normalizing plasmid, 20-μl aliquots of whole cell extracts were analyzed sequentially for firefly and Renilla luciferase activities using a dual luciferase assay kit (Promega), and the ratio of firefly to Renilla RLU was taken to represent the normalized (firefly) luciferase activity.

Western Immunoblotting—Cytosolic extracts (10 μg of protein) were fractionated by SDS-polyacrylamide gel electrophoresis (8% separating gel) and transferred to nitrocellulose membranes (Schleicher and Schuell). IκBα was detected using rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology) at 1:500 dilution for 2 h at 37 °C and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at 1:3,000 dilution for 1 h at room temperature. Detection was with enhanced chemiluminsescence, according to the manufacturer's directions (Amersham Pharmacia Biotech).

Data and Statistical Analysis—Significance of the differences between the experimental conditions were determined by paired two sample Student's *t* test (Microsoft EXCEL). The data presented are the means ± S.D.

RESULTS

PG490 Inhibits IL-2 Expression by Normal Human Peripheral Blood Lymphocytes—PG490 potently inhibits IL-2 expression by human PBLs stimulated by PMA + anti-CD3 (PMA/αCD3, Fig. 1*A*, *rows 5–8 versus row 4*), and by PMA + ionomycin (Fig. 1*A*, *rows 15–17 versus row 14*). The IC_{50} for PG490 inhibition of PMA/αCD3-stimulated IL-2 expression is

[4] J. Fidler and R.-L. Jin, Pharmagenesis, private communication.

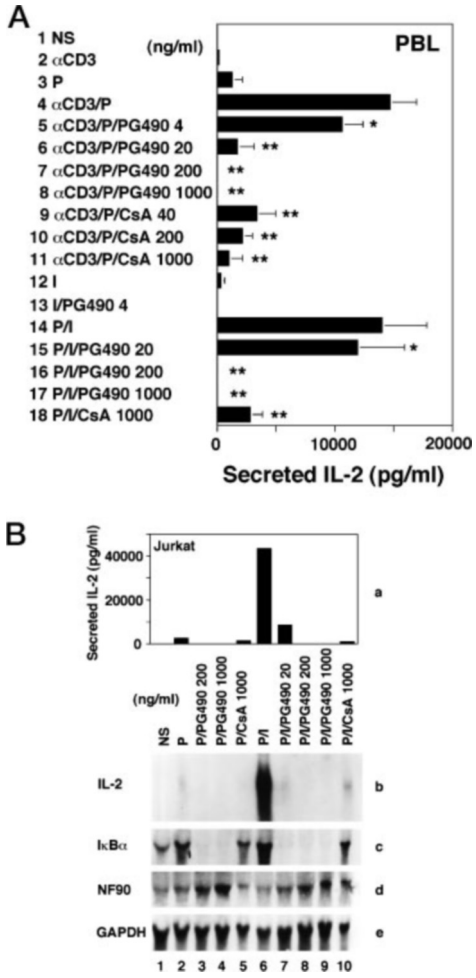

FIG. 1. **PG490 inhibition of T-cell expression of IL-2.** *A,* PG490 inhibits IL-2 expression by normal human peripheral blood lymphocytes (*PBL*) stimulated with PMA, αCD3, and with P/I. *B*, PG490 inhibits Jurkat T-cell expression of IL-2 protein (*panel a*), IL-2 mRNA (*panel b*) and IκBα mRNA (*panel c*), and increases expression of NF90 mRNA (*panel d*) with no changes of GAPDH mRNA (*panel e*). Normal human peripheral blood lymphocytes or Jurkat T-cells were stimulated for 12 h with PMA (*P*) (20 ng/ml), anti-CD3 (αCD3, clone HIT3a, 10 μg/ml in solution, Pharmingen), ionomycin (*I*) (2 μM), P/αCD3, or P/I in the presence of the indicated concentrations of PG490 or CsA. Cell supernatants were assayed for IL-2 by ELISA (Immunotech, Westbrook, ME), and Jurkat total cellular RNA was fractionated and analyzed by Northern hybridization. The PBL-IL-2-ELISA data represent the means ± S.D. from four independent experiments; statistically significant inhibitions compared with no drug treatment are indicated (*$p < 0.05$; **$p < 0.01$).

approximately 10 ng/ml (28 nM). PG490 at 200 ng/ml (560 nM) causes more complete inhibition of PMA/αCD3-stimulated IL-2 expression than CsA at 1,000 ng/ml (832 nM, Fig. 1*A*, *row 7 versus row 11*). Ionomycin stimulation alone causes minimal induction of IL-2 expression, which is inhibited by the lowest dose of 4 ng/ml PG490 (Fig. 1*A*, *rows 12* and *13*). The IC_{50} for PG490 inhibition of PMA/Iono-stimulated IL-2 expression is approximately 40 ng/ml (112 nM).

PG490 Inhibits Jurkat T-Cell Cytokine and Cytokine Regulator Gene Expression Differently Than CsA—Similar to the result in human PBLs, we show that PG490 potently inhibits Jurkat T-cell expression of IL-2 (Fig. 1*B*, *panel a*). Stimulation with PMA/Iono bypasses membrane signaling events of T-cell activation (27) and strongly induces IL-2 protein and mRNA expression (Fig. 1*B*, *panels a* and *b*, *lane 6*). PG490 at 20 ng/ml (56 nM) causes over 80% inhibition of PMA/Iono-stimulated IL-2 protein and mRNA expression (Fig. 1*B*, *panel a* and *b*, *lane 7 versus lane 6*). PG490 at 200 ng/ml more completely suppresses Jurkat T-cell IL-2 protein and mRNA expression stimulated by PMA and by PMA/Iono than 1,000 ng/ml of CsA (Fig. 1*B*, *panels a* and *b*, *lanes 3* and *5 versus lane 2*, and *lanes 8* and *10 versus lane 6*).

Using Northern hybridization analysis, we show that PG490 has distinct effects on the mRNA expression of cytokine regulators IκBα and NF90 (Fig. 1*B*, *panels c* and *d*). We observe constitutive expression of IκBα mRNA (28) (Fig. 1*B*, *panel c*, *lane 1*), and this expression is further stimulated by PMA and PMA/Iono (Fig. 1*B*, *panel c*, *lanes 2* and *6*). PG490 potently inhibits IκBα mRNA expression (Fig. 1*B*, *panel c*, *lanes 3*, *4*, and *7-9*), whereas 1,000 ng/ml CsA shows only limited inhibition (Fig. 1*B*, *panel c*, *lanes 5* and *10*). In contrast, mRNA expression for NF90, a transcriptional regulator of IL-2 gene expression (13), is significantly induced in the presence of 200 and 1,000 ng/ml PG490 (Fig. 1*B*, *panel d*, *lanes 3* and *4 versus lane 2* and *lanes 8* and *9 versus lane 6*). Finally, the levels of GAPDH mRNA are unaltered in response to stimulation and drug treatment (Fig. 1*B*, *panel e*). Our observations that PG490 causes more complete inhibition of IL-2 expression than CsA and that PG490 but not CsA inhibits IκBα mRNA expression and enhances NF90 mRNA expression establish that PG490 modulates gene expression through distinctly different mechanisms than CsA.

PG490 Inhibits IL-2 Gene Transcription through Mechanisms Different Than CsA—We next demonstrated that PG490 inhibits activation of an IL-2 luciferase reporter gene transfected transiently and stably into Jurkat T-cells (Fig. 2), and this result implies that PG490 inhibits IL-2 expression at the level of transcriptional activation of the IL-2 gene.

We used the IL-2 luciferase assay to test the ability of PG490 to inhibit the CsA-resistant pathway of T-cell activation achieved by stimulation with PMA in combination with anti-CD28 monoclonal antibody (18). We stimulated Jurkat T-cells that stably express the IL-2 luciferase reporter gene with either PMA + anti-CD28 antibody (PMA/αCD28), or with PMA/Iono, each in the presence of PG490 or CsA (Fig. 2*A*). For T-cells stimulated with PMA/Iono (Fig. 2*A*, *left panel*), the IC_{50} is approximately 20 ng/ml (56 nM) for PG490 and approximately 3 ng/ml (2.5 nM) for CsA inhibition of IL-2 transcription. In contrast, for T-cells stimulated with PMA/αCD28 (Fig. 2*B*, *right panel*), the IC_{50} is approximately 50 ng/ml for PG490 inhibition of IL-2 transcription, and CsA is completely ineffective in inhibiting this pathway of stimulation. At the two lower doses of PG490 but not of CsA, we observed modest increases in IL-2 luciferase activity before inhibition occurs at the higher doses. These results demonstrate that although CsA is more potent than PG490 in inhibiting PMA/Iono-stimulated IL-2 luciferase activity, PG490 is capable of inhibiting T-cell activation triggered through a costimulatory receptor, a situation in which CsA is ineffective.

To explore further the mechanistic differences between PG490 and CsA and FK506 inhibition of IL-2 transcription, we tested the effects of calcineurin overexpression on sensitivity to inhibition by these immunosuppressant drugs (Fig. 2*B*) Overexpression of calcineurin in Jurkat T-cells confers relative resistance to the inhibitory effects of CsA and FK506 upon transcriptional activation of the IL-2 gene (29, 30). We transiently cotransfected Jurkat T-cells with the IL-2 luciferase reporter plasmid together with either an empty expression vector or with expression vectors encoding calcineurin A and B subunits (Fig. 2*B*). The transfected T-cells were stimulated with PMA/Iono in the presence of increasing doses of PG490 (Fig. 2*B*, *left panel*), CsA (Fig. 2*B*, *center panel*), or FK506 (Fig. 2*B*, *right panel*). There is no shift in the dose-inhibition curve of IL-2

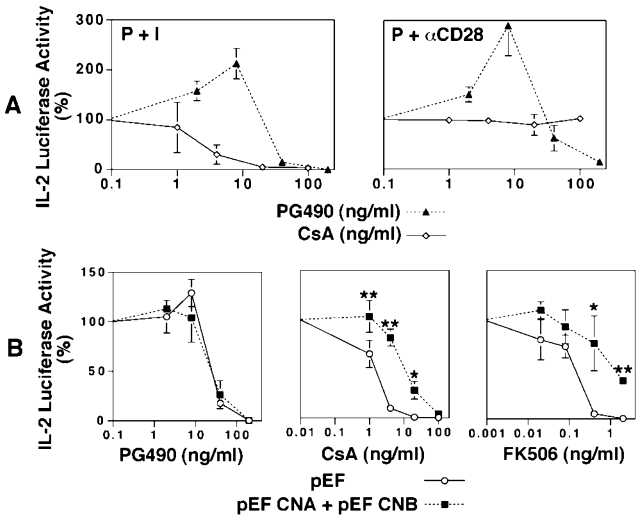

FIG. 2. **PG490 inhibits IL-2 transcriptional activation through mechanisms different from CsA.** *A,* PG490 but not CsA inhibits T-cell activation stimulated through costimulatory receptor CD28. Jurkat T-cells that stably express an IL-2 luciferase reporter plasmid were stimulated for 15 h with either P/I or P+αCD28 monoclonal antibody (YTH913.12, BIOSOURCE) then whole cell extracts were assayed for luciferase activity. The mean luciferase activity in nonstimulated cells was ~2,000 RLU, in P/I-stimulated cells was ~76,000 RLU, and in P/αCD28-stimulated cells was ~3,400 RLU. For each independent experiment, the P/I-stimulated and the P/αCD28-stimulated luciferase values with zero drug present were set as 100% (these values are not plotted because the *x* axis is on a log scale). The data shown are the means ± S.D. from three independent experiments. *B*, PG490 inhibition of T-cell activation is unaffected by overexpression of calcineurin. Jurkat T-cells were transiently transfected with an IL-2 luciferase reporter construct, together with either empty expression vector (*filled squares*), or with expression vectors directing synthesis of calcineurin A and calcineurin B (*empty squares*). 20 h following transfection, cells were stimulated with P/I for 6 h in the presence of PG490, CsA, or FK506 at the indicated doses then whole cell extracts were prepared for analysis of luciferase activity. The mean luciferase activity for the nonstimulated condition was 300 RLU and for the P/I-stimulated condition was ~300,000 RLU. For each experiment, the P/I-stimulated value with zero drug present was set at 100% (this point is not plotted because the *x* axis is on a log scale). The data shown are the means ± S.D. from three independent experiments. Statistically significant differences in IL-2 transcription at the same doses of CsA or FK506 following transfection of calcineurin expression plasmids are indicated ($*p < 0.05$; $**p < 0.01$).

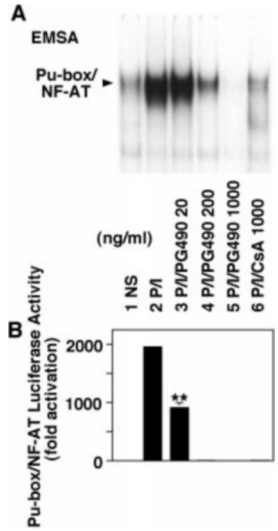

FIG. 3. **PG490 inhibits purine-box/NF-AT transcriptional activation and induced purine-box/ARRE/NF-AT DNA binding.** *A,* EMSA analysis showing inhibition of induced purine-box/ARRE/NF-AT DNA binding activity by PG490. Jurkat T-cells were stimulated for 3 h with P/I in the presence of the indicated concentrations of PG490 then nuclear extracts were prepared, and DNA binding activities were analyzed by EMSA. *B*, inhibition of purine-box/NF-AT transcriptional activation by PG490. Jurkat T-cells that stably express a purine-box/NF-AT luciferase reporter construct were stimulated for 6 h with P/I in the presence of the indicated doses of PG490. The mean luciferase activity in nonstimulated cells was 550 RLU and in P/I-stimulated cells was ~1,100,000 RLU. The data shown are the means ± S.D. from three independent experiments. Statistically significant inhibitions compared with no drug treatment are indicated ($*p < 0.05$; $**p < 0.01$). *Pu*, purine.

transcription by PG490 conferred by overexpression of calcineurin (Fig. 2*B*, *left panel*). In contrast, overexpression of calcineurin shifts the IC_{50} for CsA inhibition of IL-2 transcription from approximately 2 to 10 ng/ml (Fig. 2*B*, *center panel*), and shifts the IC_{50} for FK506 inhibition of IL-2 transcription from approximately 0.2 to 1 ng/ml (Fig. 2*B*, *right panel*). These results demonstrate that PG490 inhibits IL-2 transcription through mechanisms distinct from CsA and FK506, and which probably do not involve calcineurin.

PG490 Inhibits Transcriptional Activation and Also DNA Binding of the Regulator of the Purine-box/ARRE/NF-AT Target Site in the IL-2 Enhancer—After demonstrating that PG490 inhibits IL-2 expression at the level of transcription, we next investigated the effects of PG490 on the induction of DNA binding activity and transcriptional activation of specific factors controlling the purine-box/ARRE/NF-AT and NF-κB target sequences in the IL-2 enhancer (reviewed in Ref. 11). We used EMSA to analyze the effects of PG490 on the PMA/Iono-induced Jurkat T-cell purine-box/ARRE/NF-AT DNA binding activity (Fig. 3*A*). PG490 inhibits the induction of the purine-box/ARRE·EMSA complex with an IC_{50} slightly below 200 ng/ml (Fig. 3*A*, *lane 4 versus lane 2*). At 1,000 ng/ml PG490, the PMA/Iono-induced purine-box/ARRE·EMSA complex is undetectable (Fig. 3*A*, *lane 5*) and is therefore weaker than the EMSA complex constitutively present in nonstimulated cells (Fig. 3*A*, *lane 1*), and weaker than the complex induced in the presence of 1,000 ng/ml CsA (Fig. 3*A*, *lane 6*). PG490 is more potent in inhibiting PMA/Iono-stimulated transcriptional activation of a purine-box/NF-AT luciferase reporter gene with an IC_{50} of approximately 20 ng/ml (Fig. 3*B*, *lane 3 versus lane 2*). Taken together, these results demonstrate that PG490 inhibits both transcriptional activation and also DNA binding of the regulator operating at the purine-box/ARRE/NF-AT target sequence and that the predominant site of signaling inhibition is at the level of transcription after specific binding to DNA.

PG490 Inhibits Transcriptional Activation and not DNA Binding of NF-κB—We investigated the effects of PG490 on NF-κB signaling in Jurkat T-cells (Fig. 4). We show constitutive expression of the NF-κB p65 anchoring protein, IκBα, in the cytoplasm of nonstimulated Jurkat T-cells (Fig. 4*A*, *lane 1*), and IκBα expression decreases following stimulation with PMA (Fig. 4*A*, *lane 2 versus lane 1*). PG490 at 200 and 1,000 ng/ml causes nearly complete inhibition of IκBα protein expression in PMA-stimulated T-cells (Fig. 4*A*, *lanes 4* and *5*), and this result correlates with PG490 inhibition of IκBα mRNA expression (Fig. 1*B*, *panel c*, *lanes 3* and *4*).

At the level of NF-κB DNA binding, stimulation of Jurkat T-cells with PMA induces the appearance of a new band (NF-κB complex, Fig. 4*B*, *lanes 2–6*). The presence of the NF-κB p65 subunit within this complex is established by the quantitative supershift of the inducible complex with an antibody to p65 (Fig. 4*B*, *lanes 7–12*). PG490 at 20 ng/ml causes no significant effect on the NF-κB·EMSA complex (Fig. 4*B*, *lane 3 versus lane 2*), and PG490 at 200 and 1,000 ng/ml causes a significant increase in the strength of the NF-κB complex (Fig. 4*B*, *lanes 4* and *5 versus lanes 2* and *10*, *lane 11 versus lane 8*). We propose that the increase in NF-κB DNA binding activity

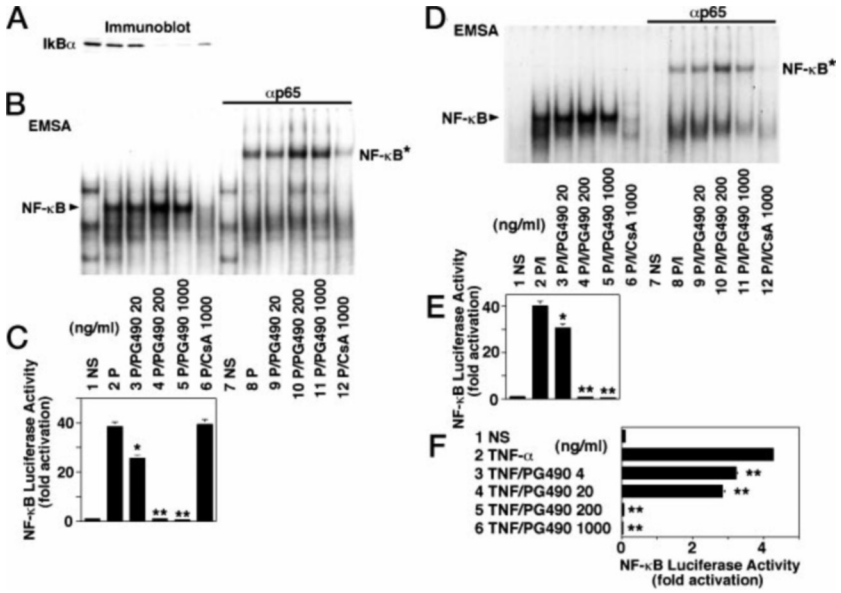

FIG. 4. **PG490 inhibits NF-κB transcriptional activation with no inhibition of induced NF-κB DNA binding.** *A*, Western immunoblot of cytoplasmic IκBα expression following PMA stimulation and effects of PG490. *B*, EMSA analysis of nuclear NF-κB DNA binding activity following PMA stimulation and effects of PG490. Jurkat T-cells were stimulated with PMA (20 ng/ml) for 3 h in the presence of the indicated concentrations of PG490 or CsA and then cytoplasmic and nuclear extracts were prepared and used for Western and EMSA analyses. Supershift analyses were performed by the addition of 2 μl of IgG against NF-κB p65 (sc-109, Santa Cruz Biotechnology). *C*, inhibition of PMA-stimulated NF-κB transcriptional activation by PG490. The mean luciferase activity per 20 μg of protein in NS cells was 34,500 RLU and ~1,320,000 RLU in PMA-stimulated cells. *D*, EMSA analysis of nuclear NF-κB DNA binding activity following P/I stimulation and effects of PG490. The nuclear extracts analyzed are the same as in Fig. 3*A* and were prepared from Jurkat T-cells stimulated for 3 h with PMA/Iono. *E*, inhibition of PMA/Iono-stimulated NF-κB transcriptional activation by PG490. The mean luciferase activity per 20 μg of protein in NS cells was 60,595 RLU and ~2,439,652 RLU in PMA/Iono-stimulated cells. *F*, inhibition of TNF-α-stimulated NF-κB transcriptional activation by PG490. The mean luciferase activity per 20 μg of protein in NS cells was 82,147 RLU and ~351,202 RLU in TNF-α-stimulated cells. For the NF-κB-luciferase experiments in *C*, *E,* and *F*, Jurkat T-cells that stably express an NF-κB luciferase reporter construct were stimulated for 6 h either with PMA (20 ng/ml), P/I (2 μM), or TNF-α (20 ng/ml) in the presence of the indicated doses of PG490 or CsA. The data shown are the means ± S.D. from three independent experiments. Statistically significant inhibitions compared with no drug treatment are indicated (*$p < 0.05$; **$p < 0.01$).

which we observe at 200 and 1,000 ng/ml PG490 occurs as a consequence of PG490 inhibition of IκBα expression, which likely allows increased nuclear translocation of p65.

Despite the development of a strong NF-κB DNA binding complex in the nucleus of T-cells stimulated with PMA in the presence of 200 ng/ml PG490 (Fig. 4*B*, *lane 4*), we observe nearly 90% inhibition of transcriptional activation of an NF-κB luciferase reporter gene (Fig. 4*C*, *lane 4*). CsA at 1,000 ng/ml shows no significant inhibition of PMA-stimulated NF-κB transcription (Fig. 4*C*, *lane 6*), which is expected, because CsA acts predominantly to inhibit calcium-mediated activation pathways. The inhibitory effects of PG490 on NF-κB-mediated transcriptional activation were identical using stably or transiently transfected cells, or when using a consensus immunoglobulin κ light chain NF-κB binding site monomer in the context of the minimal IL-8 promoter, or the IL-2 NF-κB site trimer in the context of the minimal IL-2 promoter (data not shown).

Although PMA stimulation alone activates NF-κB DNA binding and transcription (Fig. 4, *B* and *C*), substantial expression of IL-2 mRNA and protein requires the additional calcium mobilization achieved following ligation of the T-cell receptor (Fig. 1*A*) or with ionophore (Fig. 1, *A* and *B*). We, therefore, also examined the effects of PG490 upon NF-κB signaling triggered with PMA + ionomycin (Fig. 4, *D* and *E*). Similar to the results observed with PMA stimulation, PMA/Iono stimulation causes the induction of specific nuclear NF-κB DNA binding activity (Fig. 4*D*, *lanes 2* and *8*), which increases when cells are stimulated in the presence of PG490 (Fig. 4*D*, *lanes 3–5* and *9-11*). Again, a specific antibody to NF-κB p65 causes a quantitative supershift of the inducible DNA binding complex (Fig. 4*D*, *lanes 7–12*). Cells stimulated with PMA/Iono in the presence of CsA show much less induction of NF-κB DNA binding activity than cells stimulated in the presence of PG490 (Fig. 4*D*, *lane 6 versus lanes 3–5*), underscoring the different mechanisms of action of these immunosuppressant drugs. Despite the development of strong NF-κB DNA binding activity, PG490 causes near complete inhibition of PMA/Iono-stimulated NF-κB transcriptional activation with an IC_{50} of approximately 50 ng/ml (Fig. 4*E*, *lanes 3–5*). Transcriptional activation of NF-κB stimulated by TNF-α is also inhibited by PG490, with an IC_{50} of approximately 50 ng/ml (Fig. 4*F*, *rows 3-6*).

PG490 nearly completely inhibits NF-κB-mediated transcription triggered by all stimuli tested (PMA, PMA/Iono, TNF-α, and IL-1β data not shown). Taken together, our results demonstrate that PG490 inhibits NF-κB transcriptional activation at a step after specific binding to DNA.

PG490 Inhibits Activation through NF-κB Transactivation Domains TA1 and TA2—Because PG490 inhibits NF-κB transcriptional activation without inhibiting nuclear NF-κB DNA binding activity (Fig. 4, *B–E*), we next investigated whether PG490 can inhibit transcriptional activation through a chimeric transcription factor in which the transactivating domain of p65 is fused to the yeast GAL4 DNA binding domain. Schmitz and Baeuerle (25) identified that the C-terminal portion of NF-κB p65 contains two transactivation domains, TA1 and TA2, and showed that chimeric fusion proteins with the TA1 (p65 amino acids 522–551) or TA1 + TA2 (p65 amino acids 286–521) domains fused to the DNA binding domain of GAL4 (amino acids 1–147) confer PMA-inducible transcriptional activation onto a GAL4 reporter gene (25, 31). We transiently transfected Jurkat T-cells with a GAL4 response element-luciferase reporter construct, together with expression constructs encoding either the GAL4 DNA binding domain alone (GAL4DB), GAL4DB-p65TA1, or GAL4DB p65(TA1+ TA2). After recovery from the transfection, the cells were stimulated with PMA, in the presence of increasing doses of PG490. The

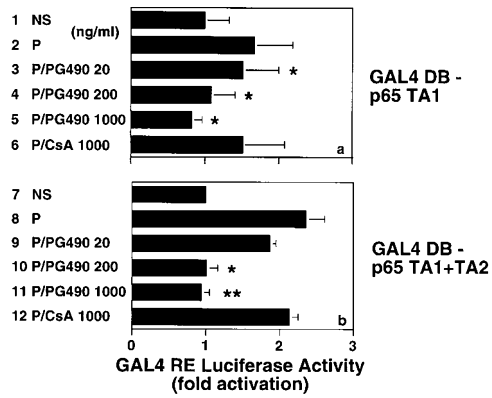

FIG. 5. **PG490 inhibits activation through chimeric transcription factors GAL4DB-NF-κBTA1 and GAL4DB-NF-κB{TA1+TA2}.** Jurkat T-cells were transiently transfected with 2 μg of GAL4RE luciferase reporter plasmid and 4 μg of expression plasmids for either GAL4DB-p65 TA1 or GAL4DB-p65{TA1+TA2}. In *panel a*, cells were also cotransfected with 1 μg of pEF Renilla luciferase plasmid to normalize for transfection efficiency. 20 h after transfection, cells were stimulated for 6 h with PMA then whole cells extracts were assayed for luciferase activity (*panel b*); in *panel a*, both firefly and Renilla luciferase were assayed using a dual luciferase assay kit (Promega). In *panel a*, the mean normalized luciferase activity in NS cells was 6,600 RLU and ~9,600 RLU in PMA-stimulated cells; in *panel b*, the mean luciferase activity per 20 μg of protein in NS cells was 258,000 RLU and ~600,000 RLU in PMA-stimulated cells. The data shown represent the means ± S.D. from five (*panel a*) and three (*panel b*) independent experiments. Statistically significant inhibitions of the PMA-stimulated condition by PG490 are indicated (*$p < 0.05$; **$p < 0.01$).

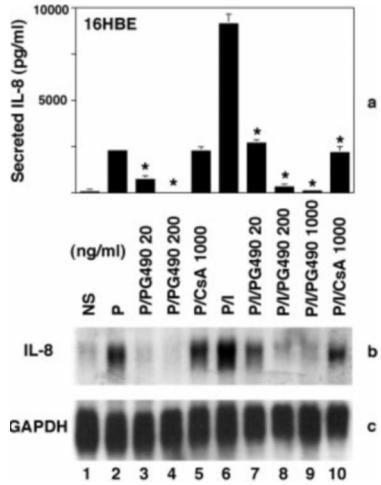

FIG. 6. **PG490 inhibits bronchial epithelial cell IL-8 expression.** 16HBE cells were treated for 6 h with the indicated stimulants and immunomodulating drugs then secreted IL-8 was analyzed by ELISA (Immunotech), and total RNA was prepared and analyzed for IL-8 and GAPDH mRNA expression by Northern hybridization. The ELISA data are the means ± S.D. from four independent experiments; statistically significant inhibitions by PG490 or CsA are indicated (*$p < 0.001$).

expression construct for GAL4DB alone shows no transactivation of the GAL4 response element (GAL4RE, data not shown), demonstrating that Jurkat T-cells contain no endogenous activators of the GAL4RE reporter. There is constitutive transcription by the chimeric transcription factor, GAL4DB-p65 TA1, and this transcription is enhanced approximately 1.6-fold following stimulation with PMA, and the PMA enhancement of transcription is inhibited by PG490 (Fig. 5, *panel a*). We observed a greater signal with a similar pattern of regulation using the chimeric transcription factor GAL4DB p65(TA1 + TA2); the constitutive transcription is enhanced approximately 2.3-fold following stimulation with PMA, and the PMA enhancement of transcription is inhibited by PG490 (Fig. 5, *panel b*). Notably, PG490, but not CsA, inhibits transcription through the C-terminal transactivation domains TA1 and TA1 + TA2 of p65 (Fig. 5, *panels a* and *b*). The IC_{50} for PG490 inhibition of the chimeric transcription factors is approximately 50 ng/ml, similar to the IC_{50} for PG490 inhibition of endogenous NF-κB activity (Fig. 4, *C*, *E*, and *F*).

PG490 Inhibits IL-8 Expression in 16HBE Human Bronchial Epithelial Cells—NF-κB is important for transcriptional activation of cytokine genes such as IL-8 in nonlymphoid cells including epithelial and endothelial cells (32). We therefore examined the effects of PG490 on expression of IL-8 protein and mRNA in 16HBE transformed human bronchial epithelial cells (Fig. 6). PG490 at 20 ng/ml inhibits PMA-stimulated IL-8 protein and mRNA expression more effectively than CsA at 1,000 ng/ml (Fig. 6, *panels a* and *b*, *lane 3 versus lane 5*), and PG490 at 200 and 1,000 ng/ml inhibits PMA/Iono-stimulated IL-8 expression more effectively than 1,000 ng/ml CsA (Fig. 6, *lanes 8* and *9 versus lane 10*). These results demonstrate that PG490 inhibits inflammatory cytokine gene expression in epithelial cells, as well as in lymphoid cells, and the mechanism of inhibition is distinctly different from CsA and probably involves transcriptional inhibition of NF-κB.

DISCUSSION

PG490, which is pure triptolide, inhibits T-cell activation and early cytokine gene transcription in T-cells and epithelial cells through mechanisms that are different from CsA and FK506. PG490 inhibits transcriptional activation at the purine-box/ARRE/NF-AT and NF-κB target sequences after specific binding to DNA.

In previous studies of the T2 ethyl acetate extract of Tripterygium, Tao *et al.* (9) inferred that the immunosuppressive properties of T2 are due to triptolide and tripdiolide and showed that T2 completely suppressed mitogen-stimulated IL-2 and interferon-γ expression in T-cells but showed only limited inhibition of IL-6, IL-2 receptor, and transferrin receptor expression (10). IL-2 expression was inhibited by T2 at the level of transcription, as revealed by an IL-2 reporter gene assay in Jurkat T-cells (10). The *in vitro* phosphatase activity of calcineurin was not significantly inhibited by immunosuppressive doses of T2, triptolide, or tripdiolide (10).

In our experiments, we studied PG490, which is 97% pure triptolide, in contrast to the T2 ethyl acetate extract that contains approximately 5% triptolide. We found that PG490 potently inhibits IL-2 protein and mRNA expression, and this inhibition occurs at the level of IL-2 transcription, and our results are consistent with the characterization of T2 (10). Using an *in vivo* assay, we found that overexpression of calcineurin in Jurkat T-cells does not alter sensitivity to inhibition by PG490 while conferring a 5-fold relative resistance to the inhibitory effects of both CsA and FK506. This result establishes that PG490 inhibits IL-2 transcription through mechanisms distinct from CsA and FK506 and which likely do not involve calcineurin. In addition, we show that PG490 but not CsA inhibits IL-2 transcription stimulated by PMA and monoclonal antibody YTH913.12 to the costimulatory receptor, CD28. In contrast to our results, Tao *et al.* (10) found that a maximal dose of T2 (2 μg/ml) was unable to inhibit IL-2 secretion by purified T-cells stimulated with PMA + monoclonal antibody 9.3 to CD28. We believe that this difference in results can most likely be attributed to our use of pure triptolide compared with the use of the T2 mixture (10), which contains only a fraction of triptolide. The other compounds present in T2 may be physiologically inactive, in which case their presence

might simply limit the dose availablity of triptolide; alternatively, other compounds present in T2 might modulate the immunosuppressive activities of T2 so that it becomes ineffective in comparison to pure triptolide in inhibiting T-cell activation through costimulatory receptors.

The principal regulators of IL-2 transcriptional activation operate at the purine-box/ARRE/NF-AT and NF-κB target DNA sequences (11). We demonstrate that PG490 inhibits purine-box/ARRE/NF-AT signaling both at the level of transcriptional activation and also at the level of specific binding to DNA. The IC_{50} for PG490 inhibition of PMA + ionomycin(P/I)-stimulated purine-box/NF-AT transcriptional activation is ~20 ng/ml, whereas the IC_{50} for PG490 inhibition of the induced specific purine-box/ARRE/NF-AT DNA binding complex is ~200 ng/ml. From this result, we conclude that PG490 predominantly inhibits purine-box/NF-AT signaling at the level of transcriptional activation after specific binding to DNA.

NF-κB signaling involves stimulation-induced degradation of cytoplasmic IκB (19, 33), releasing p65 for translocation from the cytoplasm into the nucleus, where p65 interacts with p50 and binds specifically to the NF-κB target DNA sequence. Following specific binding to DNA, transcriptional activation of NF-κB is regulated through specific phosphorylation of p65 at several distinct sites (34–37). We found that PG490 potently inhibits the expression of IκBα mRNA and protein, and these decreases in IκBα protein likely serve to increase the amount of p65 released for translocation into the nucleus and specific binding to DNA. Although PG490 causes an increase in nuclear NF-κB DNA binding activity, PG490 inhibits NF-κB transcriptional activation at a step after specific binding to DNA. The IκBα enhancer contains at least two potential binding sites for NF-κB (38). We hypothesize that PG490 inhibits IκBα mRNA and protein expression through transcriptional inhibition of the IκBα gene, probably at these NF-κB target sites.

We extended our analysis of PG490 inhibition of NF-κB transcriptional activation by demonstrating that PG490 inhibits the PMA-stimulated activation of chimeric transcription factors containing the NF-κB p65 TA1 and TA2 transactivation domains. PG490 inhibits PMA-stimulated transcriptional activation through the TA1 domain, which is highly conserved between humans, mice, and *Xenopus*, and extends from amino acid 521 to the C terminus at amino acid 551 in humans. This result implies that a target of PG490 inhibition resides within these 31 amino acids. Within TA1 there are 7 serines, which are predicted to align on one side of an α helix and form a polar region believed to act similar to acidic transcriptional activators (39). These serine residues are potential phosphorylation sites for a p65 regulatory kinase stimulated by PMA. Ostrowski *et al.* (40) identified a serine kinase activity that existed in the nucleus and that copurified with p65 through two sequential ion-exchange purifications. TNF-α stimulates phosphorylation of p65 on Ser-529 within the TA1 domain, and this phosphorylation contributes to the regulation of transcriptional activation (37). Kinases that have been shown to be involved in the regulation of NF-κB transcriptional activation include protein kinase A (34), p38 mitogen-activated protein kinase (35), and casein kinase II (36), although none of these kinases have been demonstrated to phosphorylate p65 within the TA1 domain. Nuclear coactivator proteins, cAMP response element-binding protein and p300, have been shown to interact with the TA1 + TA2 domains of p65 (41) and contribute to NF-κB transcriptional activation (41, 42).

From our results, we have developed a model for the potential mechanisms of PG490 inhibition of transcriptional activation at the NF-κB and purine-box/NF-AT sites (Fig. 7). We present the NF-κB heterodimer interacting with a consensus

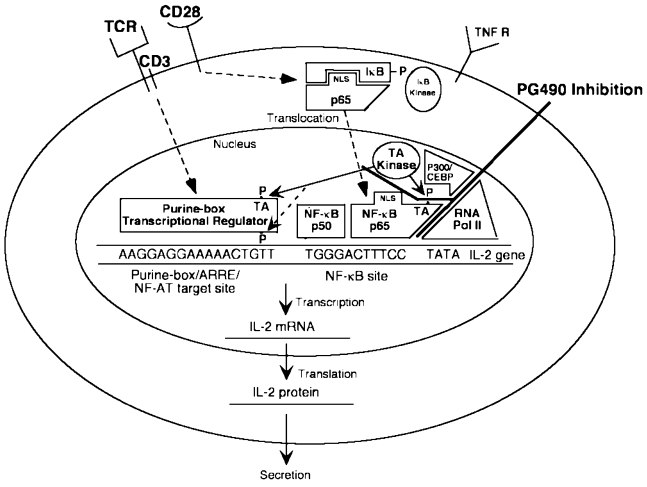

FIG. 7. **Schematic showing postulated mechanisms of PG490 inhibition of transcriptional activation at the NF-κB and purine-box/ARRE/NF-AT target DNA sequences.** T-cells stimulated at the antigen receptor and through costimulatory receptors initiate signaling pathways that lead to transcriptional activation at the purine-box/ARRE/NF-AT and NF-κB target sequences in the IL-2 enhancer. The consensus NF-κB target DNA sequence from the mouse Ig κ light chain is shown. PG490 inhibits NF-κB transcriptional activation at a step after nuclear translocation of p65 and specific binding of the p50-p65 heterodimer to DNA. PG490 also inhibits purine-box/NF-AT transcriptional activation more potently than it inhibits induced purine-box/ARRE/NF-AT DNA binding activity. A nuclear kinase that regulates transactivation of NF-κB and the purine-box regulator (TA kinase), which is a potential target of PG490 inhibition, is shown. Other potential sites of PG490 inhibition of transcriptional activation in the nucleus include recruitment of transcriptional coactivators cAMP response element-binding protein or p300 to p65 or interactions between p65 and the purine-box regulator and RNA polymerase II.

NF-κB target regulatory sequence, and the purine-box transcriptional regulator interacting with the purine-box/ARRE/NF-AT target sequence in the IL-2 enhancer. Following stimulation of cells with PMA, PMA + ionomycin, TNF, or other NF-κB-activating stimuli, there is induced degradation of IκB, releasing p65 for translocation from the cytoplasm into the nucleus. Once in the nucleus, p65 combines with p50, and the p50-p65 heterodimer binds specifically to the NF-κB target DNA sequence. We postulate that PG490 inhibition of NF-κB and purine-box/NF-AT transcriptional activation after specific DNA binding involves inhibition of regulated phosphorylation of the transactivation domains (TA) of each transcriptional regulator by a nuclear kinase, which we designate the TA kinase (Fig. 7). Because PG490 at higher doses also acts to inhibit the induced DNA binding activity of the purine-box regulator binding to the ARRE/NF-AT target sequence, we hypothesize that there is a PG490-sensitive nuclear phosphorylation step that regulates specific DNA binding of the purine-box regulator (Fig. 7). The effects of PG490 in inhibiting transcriptional activation in the nucleus after specific binding of NF-κB to DNA might also involve interference with recruitment of coactivator proteins cAMP response element-binding protein /p300 or inhibition of interactions between p65 and RNA polymerase II (Fig. 7).

Our identification that PG490 effectively inhibits T-cell activation and IL-2 gene expression triggered through pathways that are resistant to CsA suggest therapeutic utility for PG490 as an immunosuppressant capable of treating chronic allograft rejection and also graft *versus* host disease. Furthermore, signaling through members of the TNF receptor superfamily serves to simultaneously activate pathways of inflammation involving NF-κB and apoptosis involving caspases. The balance of survival over death can be influenced by the degree of acti-

vation of NF-κB (43, 44). The antiinflammatory effects of PG490 involve inhibition of NF-κB transcriptional activation after specific binding to DNA, and this inhibition of NF-κB activation probably contributes to the pro-apoptotic effects observed with PG490 (45, 46).

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