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Early Growth Response Factor-1 Mediates Prostaglandin E_2-dependent Transcriptional Suppression of Cytokine-induced Tumor Necrosis Factor-α Gene Expression in Human Macrophages and Rheumatoid Arthritis-affected Synovial Fibroblasts*

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Wissam H. Faour‡§, Nada Alaaeddine§¶, Arturo Mancini‖, Qing Wen He, Dragan Jovanovic**, and John A. Di Battista**‡‡**

*From the ‡Department of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada, the ¶Department of Pharmacology, University of Balamand, Tripoli 9616, Lebanon, and the Departments of **Medicine and ‖Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 1A1, Canada*

Tumor necrosis factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine that modulates a broad range of inflammatory and immunological processes. We have investigated the potential immunomodulatory properties of prostaglandin E_2 (PGE_2) by examining the molecular mechanism by which the eicosanoid suppresses T-cell-derived interleukin-17 (IL-17)-induced TNF-α mRNA expression and protein synthesis in human macrophages and rheumatoid arthritis-affected synovial fibroblasts. Initial studies confirmed that PGE_2 induces *egr-1* mRNA expression and protein synthesis by restricted SAPK2/p38 MAPK-dependent activating transcription factor-2 (ATF-2) dimer transactivation of the *egr-1* promoter as judged by studies using wild-type (WT) and deletion mutant *egr-1* promoter constructs, Northern and Western blotting, and standard and supershift electrophoretic mobility shift analyses. Using human leukemic monocytic THP-1 cells stably transfected with WT and dominant-negative mutant expression constructs of Egr-1, cotransfected or not with a WT pTNF−615SVOCAT construct, we observed that PGE_2 inhibition of IL-17-stimulated TNF-α mRNA expression and promoter activity was dependent on Egr-1 expression, as mutants of Egr-1, alone or in combination, markedly abrogated any inhibitory effect of PGE_2. Standard and supershift electrophoretic mobility shift analysis, signaling "decoy" overexpression studies, and pTNF−615SVOCAT promoter assays using WT and mutant promoter constructs revealed that IL-17-up-regulated promoter activity was largely dependent on ATF-2/c-Jun transactivation. PGE_2 suppression of IL-17-induced ATF-2/c-Jun transactivation and DNA binding was dependent on Egr-1-mediated inhibition of induced c-Jun expression. We suggest that *egr-1* is an immediate-early PGE_2 target gene that may be a key regulatory factor in mediating eicosanoid control of genes involved in the immune and inflammatory responses.

Inflammation and the inflammatory response represent a highly orchestrated cascade of events designed to maintain a state of homeostasis in the host following pathogenic invasion and/or tissue injury. The response involves the concerted and exquisitely timed interactions of cytokines, chemokines, growth factors, and lipid-derived mediators with inflammatory cells like macrophages, neutrophils, activated endothelial cells and fibroblasts, T- and β-lymphocytes, and mast cells (1–4). Among the most important classes of lipid-derived mediators in this regard are the prostaglandins of the E series that function as pleiotropic autacoids and exert modulatory effects on the immune and inflammatory responses in addition to acting as essential physiological regulators in virtually every tissue of the human organism (5, 6).

Prostaglandin E_2 (PGE_2)[1] is synthesized from the essential fatty acid arachidonic acid by the enzymes of the arachidonate cascade or, occasionally, by free radical-catalyzed peroxidation (7). Upon cellular activation, type IV cytosolic phospholipase A_2 translocates to the endoplasmic reticulum or outer nuclear membrane and releases arachidonic acid from the membrane lipids of most cell types (7, 8). The latter protein trafficking event is controlled by calcium fluxes and MAPK phosphorylation of cytosolic phospholipase A_2 (8). A co-localized, monotopic, integral membrane protein, prostaglandin H_2 synthase (also referred to as cyclooxygenase (COX), with two isoforms, COX-1 and COX-2), catalyzes the first committed, rate-limiting step by converting arachidonic acid to prostaglandin H_2. The latter intermediate is rapidly metabolized by cell- and tissue-specific synthases to one of several prostanoids (*e.g.* PGE_2 by prostaglandin E synthase) (9, 10).

The PGE_2 signal is transduced at the cell surface by specific cognate receptors (designated EP receptors, subtypes EP1–

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§ Both authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: Div. of Rheumatology and Clinical Immunology, Royal Victoria Hospital, McGill University Health Centre, Rm. M11.22, 687 Pine Ave. W., Montreal, Quebec H3A 1A1, Canada. Tel.: 514-842-1231 (ext. 34401); Fax: 514-289-8542; E-mail: john.dibattista@mcgill.ca.

[1] The abbreviations used are: PGE_2, prostaglandin E_2; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; CREB, cAMP response element-binding protein; ATF, activating transcription factor; AP, activator protein; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor-α; MLK, mixed lineage kinase; MKK, mitogen-activated protein kinase kinase; RA, rheumatoid arthritis; PMA, phorbol 12-myristate 13 acetate; rhIL-17, recombinant human interleukin-17; FCS, fetal calf serum; RASF, rheumatoid arthritis-affected synovial fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; WT, wild-type; PGK, phosphoglycerate kinase; CRE, cAMP response element; SRE, serum response element; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CAT, chloramphenicol acetyltransferase; SAPK, stress-activated protein kinase; LPS, lipopolysaccharide; IL, interleukin; MSK, mitogen- and stress-activated protein kinase; JNK, c-Jun N-terminal kinase.

EP4) that are characterized by an extracellular N-terminal domain, seven membrane-spanning regions containing a variable cytoplasmic loop, and a cytoplasmic C terminus. Ligand binding takes place in the membrane-spanning domains, and G-protein coupling occurs through the intracellular loops and C terminus (11–13). In general, EP2 and EP4 signal through G_s coupling, resulting in increases in cAMP levels and activated protein kinase A within seconds to minutes. The EP1 and some EP3 receptor subtypes associate with G_q and mediate increases in intracellular calcium, whereas G_i proteins reduce levels of cellular cAMP (EP3) (8, 11). For example, the C terminus of EP receptors also harbors serine/threonine residues (anywhere from 1 to 9) that, when phosphorylated by activated protein kinase A, induce receptor desensitization and signal attenuation (reviewed in Ref. 13).

In a variety of target cell types, PGE_2 modulates transcription of immediate-early genes such as *junB*, *junD*, *fosB*, *fra-1*, and c-*fos*, although the mechanistic details are completely undefined (14–16). These early gene products are transcription factors that, along with CREB, ATF-1, ATF-2, and AP-2, modify the expression of PGE_2 target genes (16, 17). The latter are genes associated with mitogenic, apoptotic, hypertrophic, and differentiation signals in many cell types, including stem cells (18, 19). Signaling cascades mediating mitogenic activity such as the Ras/Raf-1/ERK cascade are inhibited by PGE_2 secondary to phosphorylation and inactivation of Raf-1 kinase by protein kinase A (20). In addition, the p38 MAPK cascade is a primary target through which PGE_2 signaling via EP4 is manifested in human synovial fibroblasts and macrophages (21).

Preliminary data from this laboratory (23) and others (22, 24) suggest that a zinc finger transcription factor, the early growth response gene *egr-1/krox-24*, may also be a target gene of PGE_2, although detailed studies regarding the mechanism of *egr-1/krox-24* gene induction and the specificity of cell-type responsiveness were never performed. Also known as *zif268*, *egr-1/krox-24* was originally identified as a growth response gene in fibroblasts and B-cells (25, 26). The expression of Egr-1/Krox-24 is induced during differentiation of myeloid cells, nerve, and bone and has been shown to be essential and determinant for macrophage differentiation (reviewed in Ref. 26; Refs. 27 and 28). It is a strong nuclear DNA-binding protein that specifically recognizes the sequence 5′-GCG(T/G)-GGGCG-3′ and can transactivate promoters containing the appropriate cognate site. The promoters of tumor necrosis factor-α (TNF-α) (29), both chains of platelet-derived growth factor (30, 31), and transforming growth factor-β1 (30) harbor active binding/enhancer sites for Egr-1/Krox-24. Cell type determines whether Egr-1/Krox-24 acts to activate or inhibit promoter activity of target genes, suggesting that it interacts with tissue-specific factors and/or transcription factors that associate with proximal promoter elements such as Sp-1 (25, 31). In this study, we investigated in detail the regulatory mechanisms controlling PGE_2-dependent induction of the *egr-1* gene in human monocytes/macrophages and rheumatoid arthritis-transformed synovial cells. Our results indicate that PGE_2 induces Egr-1 expression through stimulation of the MLK2/MKK3/p38 MAPK cascade with ATF-2 transactivation of the *egr-1* promoter. Furthermore, using dominant-negative mutants in stably transfected cells lines, we show that PGE_2 suppresses T-cell cytokine-induced TNF-α gene expression and synthesis in human macrophages (and rheumatoid arthritis (RA)-affected synovial fibroblasts) largely through Egr-1-dependent TNF-α promoter suppression. The data suggest that PGE_2/Egr-1 disrupts essential components of the TNF-α promoter transactivation complex, *viz.* the suppression of ATF-2/c-Jun heterodimer DNA binding through inhibition of c-Jun expression.

MATERIALS AND METHODS

Chemicals—Sodium fluoride, leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride, actinomycin D, puromycin, dithiothreitol, sodium orthovanadate, and bovine serum albumin were products of Sigma. PGE_2, SB 202190, KT5720, phorbol 12-myristate 13-acetate (PMA), ionomycin, A23187, forskolin, and rolipram were purchased from Calbiochem. SDS, acrylamide, bisacrylamide, ammonium persulfate, and protein reagent were from Bio-Rad. Tris base, EDTA, $MgCl_2$, $CaCl_2$, chloroform, Me_2SO, anhydrous ethanol (95%), methanol (99%), formaldehyde, and formamide were obtained from Fisher. Recombinant human interleukin-17 (rhIL-17) was purchased from R&D Systems (Minneapolis, MN). Dulbecco's modified Eagle's medium, phosphate-free and phenol red-free Dulbecco's modified Eagle's medium, RPMI 1640 medium, TRIzol reagent, heat-inactivated fetal bovine serum, and an antibiotic mixture (10,000 units of penicillin (base) and 10,000 μg of streptomycin (base)) were products of Invitrogen.

Cell Culture—Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque density centrifugation (Amersham Biosciences) as described previously (32), and the monocytes/macrophages were allowed to adhere to plastic dishes (Flow Laboratories, McLean, VA) in RPMI 1640 medium containing L-glutamine, 10% fetal calf serum (FCS), and penicillin/streptomycin (antibiotics). THP-1 cells ($2–3 \times 10^6$ cells/ml; American Type Culture Collection, Manassas, VA), a human monocytic cell line, were cultured as suspensions in RPMI 1640 medium supplemented with 50 μM β-mercaptoethanol (23). Human RA-affected synovial fibroblasts (RASFs) were obtained from RA patients undergoing arthroplasty who were diagnosed based on criteria developed by the American College of Rheumatology Diagnostic Subcommittee for Arthritis (33). Details of RASF isolation and enrichment have been described previously (33) and were routinely cultured in Dulbecco's modified Eagle's medium, 4.5 g/liter glucose, L-glutamine, pyridoxine hydrochloride, 10% FCS, and antibiotics. NIH-3T3 and HeLa cells (American Type Culture Collection) were cultured as described above for RASFs.

Preparation of Cell Extracts and Western Blotting—Cellular proteins (50–100 μg) extracted in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, μg/ml leupeptin, μg/ml pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF) or in hot SDS-PAGE loading buffer from control and treated cells were subjected to SDS-PAGE on 8–12% gels (16 × 20 cm, final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes (Amersham Biosciences). Following blocking with 5% BLOTTO for 2 h at room temperature and washing, the membranes were incubated overnight at 4 °C with primary antibody in Tween/Tris-buffered saline containing 0.25% BLOTTO. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution) was subsequently incubated with membranes for 1 h at room temperature and washed extensively for 30–40 min with Tween/Tris-buffered saline, followed by a final rinsing with Tris-buffered saline at room temperature. Following incubation with ECL chemiluminescence reagent (Amersham Biosciences), membranes were prepared for autoradiography, exposed to Kodak X-Omat film, and subjected to digital imaging system (Alpha G-Imager 2000, Canberra Packard Canada, Mississauga, Ontario, Canada) for semiquantitative measurements as described previously (33). Anti-Egr-1, anti-Sp-1, and anti-TNF-α polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Total (independent of phosphorylation state) and anti-phospho-Elk-1 (Ser^{383}), anti-phospho-ATF-2 ($Thr^{69/71}$), anti-phospho-c-Jun ($Ser^{63/73}$), and anti-phospho-CREB-1 (Ser^{133}) polyclonal antibodies were purchased from Cell Signaling Technology (Waverly, MA).

Isolation of RNA and Northern Blotting—Total cellular RNA (1×10^6 cells = 10–20 μg of RNA) was isolated using TRIzol reagent as described previously (33). Generally, 5–20 μg of total RNA were resolved on formaldehyde-containing 1.2% agarose gel and transferred electrophoretically (30 V overnight at 4 °C) to Hybond-N^{TM} nylon membranes (Amersham Biosciences) in 0.5× Tris acetate/EDTA (pH 7). After prehybridization for 24 h, hybridizations were carried out at 50–55 °C for 24–36 h, followed by high stringency washing at 68 °C in 0.1× SSC and 0.1% SDS. The following probes, which were labeled with digoxigenin-dUTP by random priming, were used for hybridization. Human TNF-α cDNA (1.3 kb), initially cloned into the HindIII and BamHI sites of pFC54.t-1 (American Type Culture Collection), was released by digestion. Human *egr-1/krox-24* cDNA, initially cloned into the EcoRI sites of

the pMexNeoI vector (an Egr-1 expression vector; provided by Dr. D. Skup, Department of Biochemistry, University of Montreal, Montreal, Canada), contains 300 bp of 5′-noncoding region and 580 bp of 3′-noncoding region of *egr-1*. Both the c-*jun* and *junB* probes were 2.6- and 1.9-kb cDNA fragments inserted into pGEM2 (American Type Culture Collection). A 780-bp PstI/XbaI fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (1.2 kb; American Type Culture Collection) initially cloned into the PstI site of the pBR322 vector served as a control for RNA loading, as GAPDH is constitutively expressed in the cells used in these experiments. All blots were subjected to a digital imaging system (Alpha G-Imager 2000) for semiquantitative measurements, and changes in TNF-α or *egr-1* mRNA expression were always considered as the ratio of GAPDH mRNA.

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA)—Confluent control and treated cells (3–5 × 10^6 cells/well) in 4-well cluster plates were carefully scraped into 1.5 ml of ice-cold phosphate-buffered saline and pelleted by brief centrifugation. Nuclear extracts were prepared as described previously (33).

Double-stranded oligonucleotides containing wild-type (WT) and mutant sequences were obtained from Invitrogen); annealed in 100 nM Tris-HCl (pH 7.5), 1 M NaCl, and 10 mM EDTA at 65 °C for 10 min; cooled for 1–2 h at room temperature; and finally end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). The sense sequences of the oligonucleotides tested were as follows: consensus Egr-1/Krox-24, 5′-GGA TCC AGC GGG GGC GAG CGG GGG CGA-3′; consensus mutant Egr-1/Krox-24, 5′-GGA TCC AGC GGG **TA**C GAG CGG G**TA** CGA-3′; Egr-1/Krox-24 (TNF-α), 5′-GGA TCG CCC CCC GCC CCC GCG CGA-3′; mutant Egr-1/Krox-24 (TNF-α), 5′-GGA TCG CCC TAC GCC CTA GCG CGA-3′; ATF/CREB-like (*egr-1*, −131 bp), 5′-GGG GGC TTC ACG TCA CTC CGG GT-3′; mutant ATF/CREB-like (*egr-1*), 5′-GGG GGC T**GA** AGC T**AG** CTC CGG GT-3′; ATF-2/c-Jun-like (TNF-α), 5′-GAT TCA ATG AGC TCA CGG CTG TG-3′; and mutant ATF-2/c-Jun-like (TNF-α), 5′-GAT TCA A**AG ACA** TCA CGG CTG TG-3′. Binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, and 2.5 μg of poly(dI-dC). Binding reactions were conducted with 15 μg of nuclear extract and 100,000 cpm ^{32}P-labeled oligonucleotide probe at 22 °C for 20 min in a final volume of 10 μl. For supershift analysis, 2 μg of affinity-purified anti-total/phospho-CREB-1 (1:1), anti-total/phospho-ATF-2 (1:1), anti-total/phospho-c-Jun (1:1), anti-Egr-1, or anti-Sp-1 polyclonal antiserum were incubated for 10 min with the nuclear extracts prior to the addition of the radioactive probe. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis on 6% gels in a Tris borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

Constructs, Transfections, and Reporter Assays—The PGK-puro vector, WT Egr-1/Krox-24, and dominant-negative mutants (WT minus the zinc finger region and the zinc finger region only) were a kind gift from Dr. D. Skup, and the construction of the plasmids has been described previously (28). Briefly, human *egr-1/krox-24* cDNA was removed from the pMexNeoKrox-24 vector and inserted into the EcoRI site of the PGK-puro vector (puromycin for selection), which utilizes the PGK promoter to drive transcription, and thus generated the pPGK-Egr-1/Krox-24 expression plasmid. The PGK-ΔEgr-1/Krox-24 construct was prepared by digesting pPGK-Egr-1/Krox-24 with XmnI and religating, thus removing amino acids 271–364, corresponding to an essential part of the zinc finger region. The PGK-*zf*Egr-1/Krox-24 construct was obtained by preparing oligonucleotides corresponding to amino acids 210–216 and 325–332 with the 5′-nucleotide having the sequence 5′-GGG ATG-3′ to provide a site for translational initiation, an EcoRI site, and a BamHI site. The 3′-oligonucleotide has an EcoRI and XbaI site. The oligonucleotides were used to amplify the zinc finger region from pMexNeoKrox-24, and the resulting fragment was then cloned into BamHI/XbaI-digested PGK vector. Sequencing confirmed that the insert was correct and in-frame (28). Promoter constructs containing 1395 bp of the *egr-1/krox-24* promoter region along with −925, −636, −459, −252, and −77 deletion mutants were kindly provided by Dr. D. Skup, whereas the pTNF−615CAT construct was a gift from Dr. I. Kramer (Christian-Albrechts Universitat, Kiel, Germany). Site-directed mutagenesis was performed using the Transformer™ mutagenesis kit (Clontech) and involved modifying the ATF/c-Jun site at bp −99 and −100 (CA → TC) and the Egr-1 site at bp −175 and −176 (CC → GG) of the pTNF−615CAT promoter fragment (−615 to +34) and the ATF/CRE site at bp −131 and −132 (CA → TC) of the *egr-1* promoter (kindly provided by Dr. D. Skup). *cis*-Reporter plasmids containing the luciferase reporter gene driven by a basic reporter element (TATA box) fused to tandem AP-1-, ATF/CRE-, SRE-, and NF-AT-binding elements were purchased from Stratagene. Constructs containing the transactivation domains of transcription factors (*e.g.* ATF-2 and c-Jun) fused to the Gal4 DNA-binding domain and driven by the cytomegalovirus promoter (*e.g.* pFA-ATF-2) were used in decoy experiments as described previously (33). Finally, activated pMEK3 (MKK3/6) and pPKA-CAT expression plasmids (Stratagene, Upstate) were overexpressed to determine the role in *egr-1* promoter activity.

Transient transfection experiments were conducted in 4-, 6-, or 12-well cluster plates as described previously (33) for adherent cell cultures. Transfections were conducted using FuGENE™ 6 (Roche Applied Science) or Lipofectamine™ 2000 (Invitrogen) for 6 h according to the manufacturers' protocol with cells at ~30–40% confluence. Cells were re-exposed to culture medium and 1% FCS for 2 h prior to the addition of the biological effectors. Transfection efficiencies were controlled in all experiments by cotransfection with 0.5 μg of pCMV-β-gal (Stratagene), a β-galactosidase reporter vector under the control of the cytomegalovirus promoter. For stable transfections, selection was carried out with 2 μg/ml puromycin (PGK-puro vector), and experiments were conducted in the presence of a maintenance level of 0.2 μg/ml puromycin.

THP-1 cells (10-μg total plasmid concentration, 10^7 cells/0.5 ml in RPMI 1640 medium, 10% FCS, and antibiotics) were transfected by electroporation (Gene-Pulser II, Bio-Rad) using a 7-s pulse at 300 V and 960 microfarads. Where appropriate, transfectants were selected with puromycin as described previously (23, 28). The TNF-α and *egr-1* promoter activities were assessed by measuring CAT protein using enzyme-linked immunosorbent assay (Roche Applied Science) or by measuring CAT activity using [^{14}C]chloramphenicol and thin-layer chromatography, followed by enhanced autoradiography (Amersham Biosciences), according to the manufacturers' instructions. Luciferase values, expressed as enhanced relative light units, were measured in a Lumat LB 9507 luminometer (EG&G, Stuttgart, Germany) and normalized to the level of β-galactosidase activity (absorbance at 420 nm after a 24-h incubation with *o*-nitrophenyl β-D-galactopyranoside and conversion to *o*-nitrophenol) and cellular protein (bicinchoninic acid procedure, Pierce).

Statistical Analysis—All results are expressed as the mean ± S.D. or the mean and coefficient of variation of three to five separate experiments as indicated. Transfection experiments were performed in triplicate. Statistical treatment of the data was performed by parametric (Student's *t* test) or non-parametric (Mann-Whitney) analysis if gaussian distribution of the data could not be confirmed. Significance was acknowledged when the probability was <5%.

RESULTS

PGE_2 Induces egr-1 mRNA Expression and Synthesis: Time and Dose Dependence—Before examining the role of Egr-1 in PGE_2-dependent suppression of TNF-α expression, we first characterized the kinetics (and mechanism) of PGE_2 induction of the *egr-1* gene in clinically significant cell phenotypes. We performed both dose-response and time course studies using primary cell culture models consisting of human peripheral monocytes/macrophages and RASFs. As shown in Fig. 1*A*, PGE_2 induction of the *egr-1* gene was dose-dependent, and the EC_{50} for PGE_2-dependent *egr-1* mRNA expression in either monocyte/macrophage or RASF cultures was ~46 ± 12 nmol/liter (mean ± S.D., $n = 6$). For all subsequent experimentation, 2 × EC_{50} (90% response saturation) was chosen unless otherwise indicated. The PGE_2-dependent induction of Egr-1 protein followed a dose-response profile similar to that of *egr-1* mRNA expression as judged by Western blot analysis, whereas Sp-1, a GC box-binding transcription factor, was unaffected (Fig. 1*B*). Time course studies revealed rapid rises (5 min) in *egr-1* mRNA (3.8 kb) expression following PGE_2 treatment, which increased to >10-fold over control levels after 15 min and attained steady state after 30 min, followed by a precipitous and rapid decay to near control levels. Modest levels of *egr-1* mRNA were also observed after 4 h of PGE_2 stimulation (Fig. 1*C*). Specific binding to a consensus Egr-1 enhancer ^{32}P-labeled oligonucleotide was detected by 30 min and persisted up to 8 h as judged by EMSA (Fig. 1*D*, *upper* and *lower left panels*). EMSA supershift analysis confirmed the identity of the binding species as Sp-1 and Egr-1 (Fig. 1*D*, *lower right panel*). No Sp-1 oligonucleotide binding

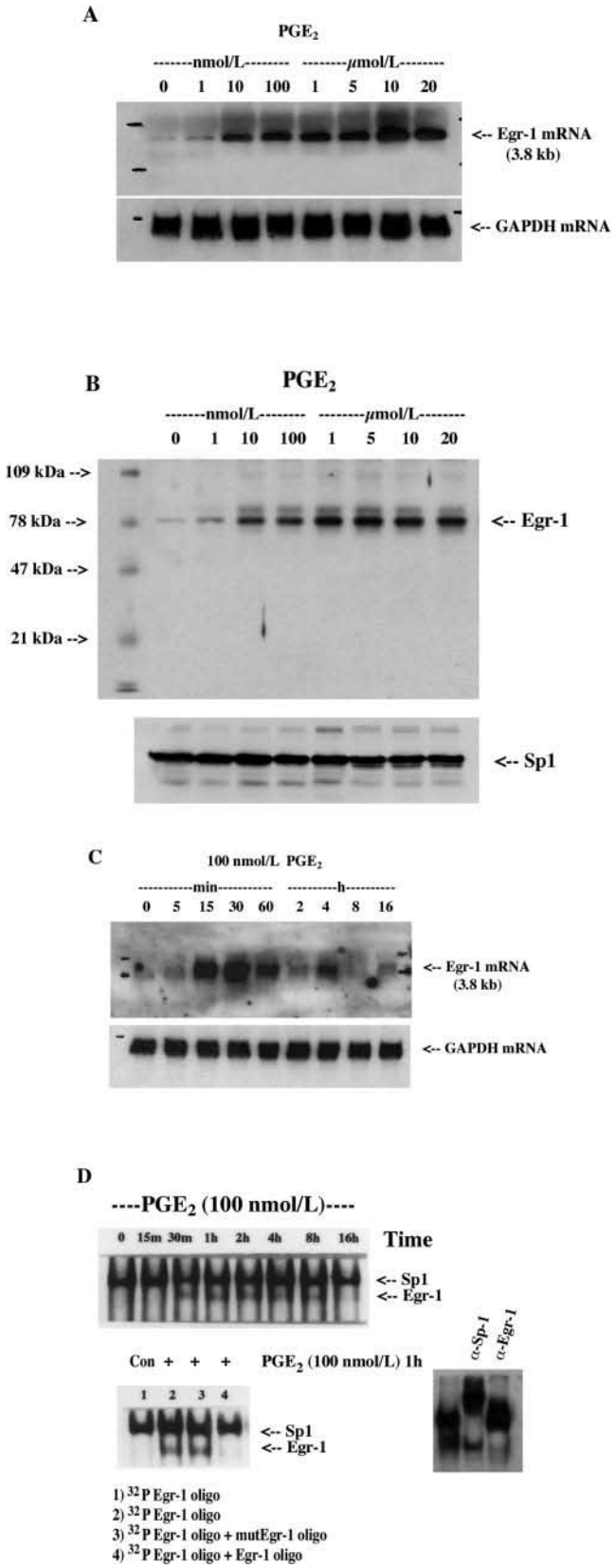

FIG. 1. **Dose response (*A* and *B*) and time course (*C* and *D*) of PGE_2 stimulation of *egr-1/krox-24* mRNA and protein.** Cultured confluent second passage human RASFs (1.2×10^6 cells in 6-well cluster plates) were preincubated for 4 h in Dulbecco's modified Eagle's medium supplemented with 1% FCS plus antibiotics at 37 °C to ensure synchrony and quiescence. Cells were then treated with increasing concentrations of PGE_2 (0–20 μmol/liter) for 30 min, and monolayers were extracted for RNA (*A*) or protein (*B*). In *C* and *D*, cells were incubated with PGE_2 for varying time periods (0–16 h). Total RNA (5 μg) was analyzed for *egr-1/krox-24* mRNA by Northern hybridization using a specific digoxigenin-labeled cDNA probe as described under "Materials and Methods" (*A* and *C*), and 50 μg of protein were analyzed for Egr-1/Krox-24 protein by Western blotting using specific polyclonal antisera for Egr-1 and Sp-1 (*B*). In *D*, 15 μg of nuclear protein were subjected to EMSA using consensus and mutant (*mut*) ^{32}P-labeled Egr-1/Krox-24 oligonucleotides, and gel supershift assays were performed with specific anti-Egr-1 and anti-Sp-1 antisera as described under "Materials and Methods." *Con*, control.

changes were observed with increased Egr-1 binding (*i.e.* no displacement) following PGE_2 treatment.

PGE_2 Induces egr-1 mRNA Expression through Activation of the MKK3/SAPK2/p38 MAPK Pathway and Transactivation of 5′-Flanking Promoter Sequences—To examine for elements of transcriptional control of PGE_2-stimulated *egr-1* expression, we conducted transient transfection analyses in THP-1 cells using a series of deletion mutants of the *egr-1* promoter. As shown in Fig. 2*A*, the *egr-1* promoter harbors a number of enhancer sites, including nested SRE, AP-1, AP-2, ATF-2/CRE, Egr-1, and other GC-rich sequences. Upon stimulation with PGE_2, elevated induction of CAT protein was observed with the pCAT−252 (SmaI) fragment (3.21 ± 0.63-fold; mean ± S.D. for three experiments in duplicate) and the −925 mutant fragment (3.85 ± 0.78-fold). For comparison purposes, we repeated the experiments with 100 nmol/liter PMA, a well known stimulator of Egr-1 (25, 26), and found maximal induction with the pCAT−459 construct (1.99 ± 0.14-fold). As shown diagrammatically in Fig. 2*A*, the pCAT−252 (SmaI) fragment has an ATF-2/CRE site at bp −138 to −131 in addition to at least one SRE site and a proximal GC box (bp −64 to −46). Because the data from Fig. 2*A* (see pCAT−77) suggest that the Sp-1 site was not responsive to PGE_2 (see also Fig. 1, *B* and *D*), we investigated whether ATF-2/CRE and/or SRE sites are crucial for promoter activation. As shown in Fig. 2*B*, promoter activation by PGE_2 was substantially decreased (>85%) when the ATF-2/CRE site was mutated. Overexpression of pMEK3 (MKK3) and, to a lesser extent, pPKA-CAT increased WT *egr-1* promoter activity (pCAT−252), whereas, as in the case with PGE_2, mutating the ATF-2/CRE site abrogated the inductive response (Fig. 2*B*). The addition of SB 202190 (100 nmol/liter) reversed the inductive effects of PGE_2 (332 ± 51 (PGE_2) *versus* 65 ± 27 (SB 202190) pg of CAT protein).

In view of the presence of the SRE sites in the pCAT−252 constructs and the possibility that the serum response factor may contribute in some way to PGE_2-dependent promoter induction, we conducted further studies with transfected reporter plasmids harboring five tandem SREs. This approach gives a very sensitive measure of cell signaling and transactivation by specific transcription factors. As shown in Fig. 2*C*, PGE_2 did not transactivate the SRE reporter as judged by measuring luciferase activity, nor did it modify serum-induced phosphorylation of Elk-1 (Fig. 2*D*), a potent SRE-binding factor and transactivator in RASFs.

We next investigated whether PGE_2 can up-regulate and phosphorylate nuclear binding proteins that bind specifically to the ATF-2/CRE site (bp −138 to −131) using Western and EMSA analyses. Previous work from our laboratory has shown that PGE_2 rapidly activates the SB 202190-sensitive SAPK2/p38 MAPK cascade through EP4 receptor cell-surface binding in inflammatory cell phenotypes (21). As shown in Fig. 3*A*, PGE_2 induced a very rapid phosphorylation of ATF-2 at $Thr^{69/71}$ and a slightly more delayed phosphorylation of ATF-1/CREB-1 at Ser^{133} (5 min). Furthermore, PGE_2 increased nuclear protein binding to the ATF-2/CRE-like oligonucleotide, and supershift analysis revealed that the principal binding species appeared to be ATF-2, with an apparently lesser

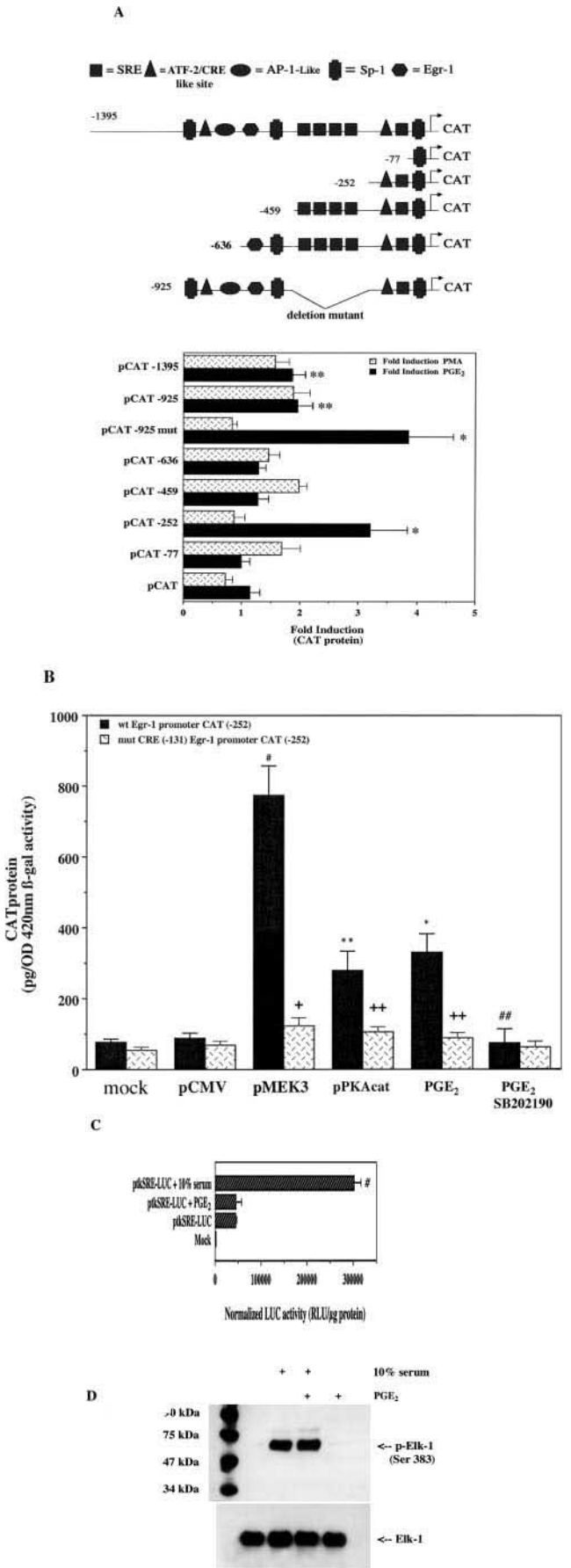

FIG. 2. **PGE_2 induction of the *egr-1* promoter: role of ATF-2/CRE and SRE.** THP-1 cells (10^7 cells/0.5 ml), cultured in RPMI 1640 medium, 10% FCS, and antibiotics, were transfected with various deletion mutants of the *egr-1* promoter by electroporation using 10 μg of total plasmid and a 7-s pulse at 300 V and 960 microfarads. Transfection efficiencies were controlled in all experiments by cotransfection with 0.5 μg of pCMV-β-gal. Cells were allowed to recover for 24 h in complete medium, after which the cells were maintained under low serum conditions (1% for 3 h) and then treated with PGE_2 or PMA (100 nmol/liter) for 6–8 h. In *A*, cells were washed and lysed, and *egr-1* promoter activity was assessed by measuring CAT protein using a specific and sensitive enzyme-linked immunosorbent assay. In *B*, cells were cotransfected with 10 μg of *egr-1* promoter construct pCAT−252 or mutant (*mut*) CRE (−131) pCAT−252 plasmid; 2 μg of pCMV, pMEK3, or pPKA-CAT; and 0.5 μg of pCMV-β-gal as described above. After 24 h, cells were maintained under low serum conditions (1% for 3 h) and then assayed for CAT protein. Cells were also treated with 100 nmol/liter PGE_2 alone or with 100 nmol/liter SB 202190 for 6 h as indicated. In *C*, cells were transfected with 10 μg of ptkSRE(5X)-LUC reporter and 0.5 μg of pCMV-β-gal as described above. Following recovery and maintenance under low serum conditions, cells were treated with vehicle, 100 nmol/liter PGE_2, or 10% serum for 6 h, and luciferase (*LUC*) values (expressed as enhanced relative light units (*RLU*)) were measured in a Lumat LB 9507 luminometer and normalized to the level of β-galactosidase (*β-gal*) activity (absorbance at 420 nm after 24 h) and cellular protein (bicinchoninic acid procedure). In *D*, cells were stimulated with vehicle (control), 10% serum, 10% serum + PGE_2 (100 nmol/liter), or PGE_2 (100 nmol/liter) for 1 h, after which whole cell lysates were subjected to Western analysis using specific antibodies to phospho-Elk-1 (Ser^{383}) and Elk-1 as described under "Materials and Methods." Probabilities were calculated by Student's *t* test and represent transfectants with the indicated promoter construct that were treated *versus* untreated (*i.e.* -fold induction). *A*: *, $p < 0.02$; **, $p < 0.05$. *B*: *, $p < 0.034$; **, $p < 0.05$; #, $p < 0.001$ *versus* pCMV; +, $p < 0.002$ *versus* pMEK3 WT *egr-1* promoter; ++, $p < 0.02$ *versus* pPKA-CAT and PGE_2 WT *egr-1* promoter; ##, $p < 0.01$ *versus* PGE_2 WT *egr-1* promoter. *C*: #, $p < 0.001$ *versus* ptkSRE(5X)-LUC.

amount of CREB-1 (Fig. 3*B*). In support of this, PGE_2 induction of *egr-1* mRNA was markedly reduced to control levels by co-incubations with SB 202190 (Fig. 3*C*) (see Ref. 33). The preferential protein kinase A inhibitor KT5720 had a more modest inhibitory effect in this regard. Phorbol ester (PMA) stimulation of *egr-1* expression was abrogated by co-incubations with calphostin C, a preferential inhibitor of the most prevalent isoforms of protein kinase C (34), but not by protein kinase A and p38 MAPK inhibitors (Fig. 3*C*).

PGE_2 Transcriptionally Suppresses rhIL-17-induced TNF-α mRNA Expression: Role of Egr-1 and AP-1 Family Members—Supported by the strong data on the mechanism of PGE_2 control of Egr-1 expression, we turned our attention to the putative role of Egr-1 in PGE_2-dependent suppression of cytokine-induced TNF-α expression and release. In this regard, a number of studies have shown that cAMP-elevating agents inhibit induced TNF-α expression, although the precise mechanism is complex and largely ill defined (reviewed in Ref. 35). Although PGE_2 can indeed elevate cAMP levels in a number of cell phenotypes, it also activates the p38 MAPK cascade and induces signaling cross-talk within the MAPK cascades (this work and Ref. 21). Recent results from our laboratory have provided physiological relevance to the latter reports to the extent that when freshly explanted osteoarthritis- or RA-affected synovial membranes were treated with preferential COX-2 inhibitors, a marked induction of TNF-α and interleukin (IL)-1β release was observed (36). Furthermore, lipopolysaccharide (LPS)-induced release of TNF-α and IL-1β was augmented >4-fold in the presence of the COX-2 inhibitor. The addition of PGE_2 returned levels to the base line, and thus, strong evidence was provided that TNF-α is an eicosanoid-dependent gene.

In this series of experiments, we used our previously described paradigm in which the CD45 T memory cell-derived pro-inflammatory cytokine IL-17 was used to stimulate TNF-α expression, synthesis, and release by human monocytes/macrophages (32). As shown in Fig. 4*A*, increased TNF-α transcripts

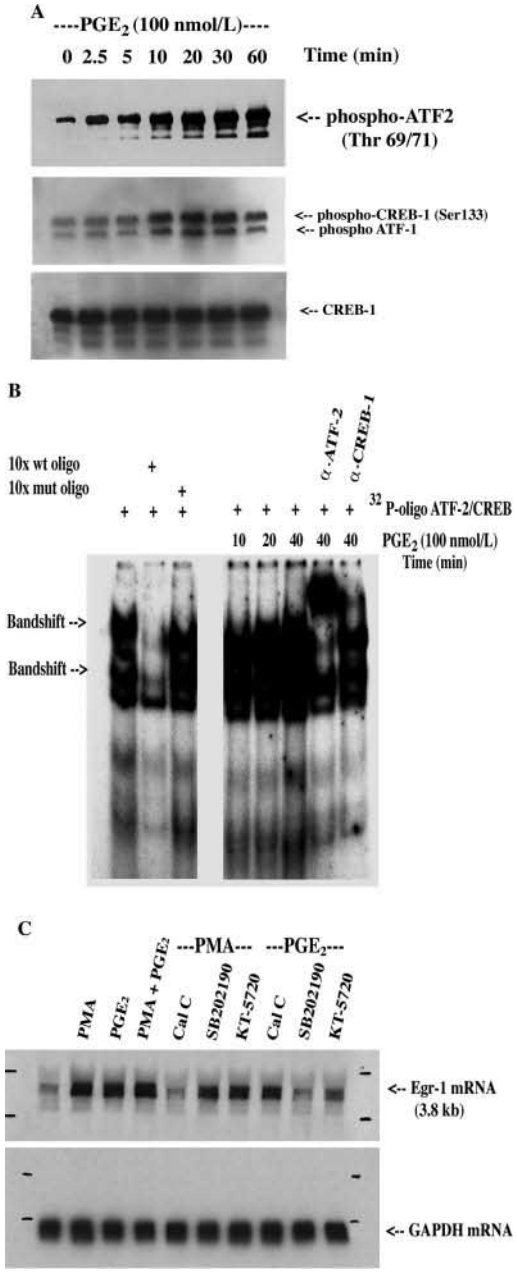

FIG. 3. **PGE_2 stimulates increased phosphorylation of ATF-2/CREB and increased binding to an *egr-1* promoter-specific ATF-2/CRE ^{32}P-labeled oligonucleotide.** In *A*, quiescent RASFs were treated with or without PGE_2 (100 nmol/liter) for varying time periods up to 60 min, after which cells were lysed, and 50 μg of protein were analyzed by Western analysis using specific antiserum to phospho-ATF-2 ($Thr^{69/71}$), phospho-ATF-1 (Ser^{133})/CREB-1, and CREB-1. In *B*, cells were stimulated with PGE_2 (100 nmol/liter) for 0–40 min, after which nuclear proteins were extracted, and EMSA and supershift analyses were performed as described under "Materials and Methods." *mut*, mutant. In *C*, cells were treated for 30 min with vehicle (*left lane*); 100 nmol/liter PMA; 100 nmol/liter PGE_2; PMA + PGE_2; PMA in the presence of 100 nmol/liter calphostin C (*Cal C*), 100 nmol/liter SB 202190, or 2 μmol/liter KT5720; or PGE_2 in the presence of calphostin C, SB 202190, or KT5720. Monolayers were extracted for RNA, and 5 μg of total RNA were analyzed for *egr-1* and GAPDH mRNAs by Northern hybridization using specific digoxigenin-labeled cDNA probes as described under "Materials and Methods."

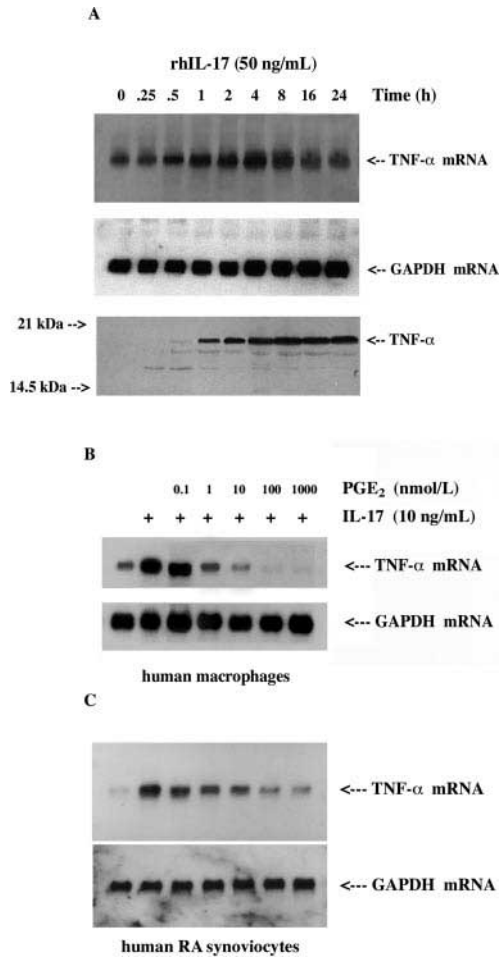

FIG. 4. **PGE_2 inhibits rhIL-17-stimulated TNF-α expression in human monocytes/macrophages and RASFs in a potent and dose-dependent fashion.** In *A*, human monocytes/macrophages (1.2×10^6 cells/well) in monolayer cultures were treated with rhIL-17 (50 ng/ml) for varying time periods (0–24 h). Cells were extracted for total RNA, and 2 μg were analyzed for TNF-α and GAPDH mRNAs by Northern hybridization using specific digoxigenin-labeled cDNA probes as described under "Materials and Methods." Spent culture medium was collected and analyzed by Western blotting for TNF-α using a specific antiserum. In *B* and *C*, human monocytes/macrophages and RASFs, respectively, were treated for 4 h with or without 50 ng/ml rhIL-17 in the absence or presence of increasing concentrations of PGE_2. Total RNAs at 2 (*B*) and 20 (*C*) μg were analyzed for TNF-α and GAPDH mRNAs by Northern hybridization.

were observed after 15–30 min, reaching steady state at ~2–4 h, followed by rapid decay. Immunoreactive TNF-α protein could be detected after ~30 min, with levels being apparently constant after 4–8 as judged by Western analysis of conditioned medium (Fig. 4*A*). Co-incubations of rhIL-17-treated human monocytes/macrophages with increasing concentrations of PGE_2 led to dose-dependent suppression of induced TNF-α mRNA levels. In both human macrophages and RA-affected synoviocytes, the IC_{50} for TNF-α suppression was ~45 ± 12 nmol/liter (mean ± S.D., $n = 4$) (Fig. 4, *B* and *C*).

Recent work from our laboratory has confirmed that PGE_2 controls gene expression at transcriptional and post-transcriptional levels (message stability) (21). As a first approach to verify whether transcriptional mechanisms are involved in the potent PGE_2-dependent inhibition of rhIL-17-induced TNF-α expression, studies were conducted with the TNF-α promoter pTNF−615SVOCAT in transiently transfected THP-1 cells treated with rhIL-17 with or without increasing concentrations of PGE_2. Using the intact promoter, rhIL-17 induced a >5-fold increase in CAT (protein) reporter synthesis, an effect abrogated by mutation of the ATF-2/c-Jun-like enhancer site (Fig. 5*A*). The addition of PGE_2 decreased rhIL-17-stimulated CAT protein synthesis (WT construct) in a dose-dependent fashion, with >95% inhibition at 100–1000 nmol/liter (Fig. 5*A*). Interestingly, mutating the Egr-1 site in the promoter did not com-

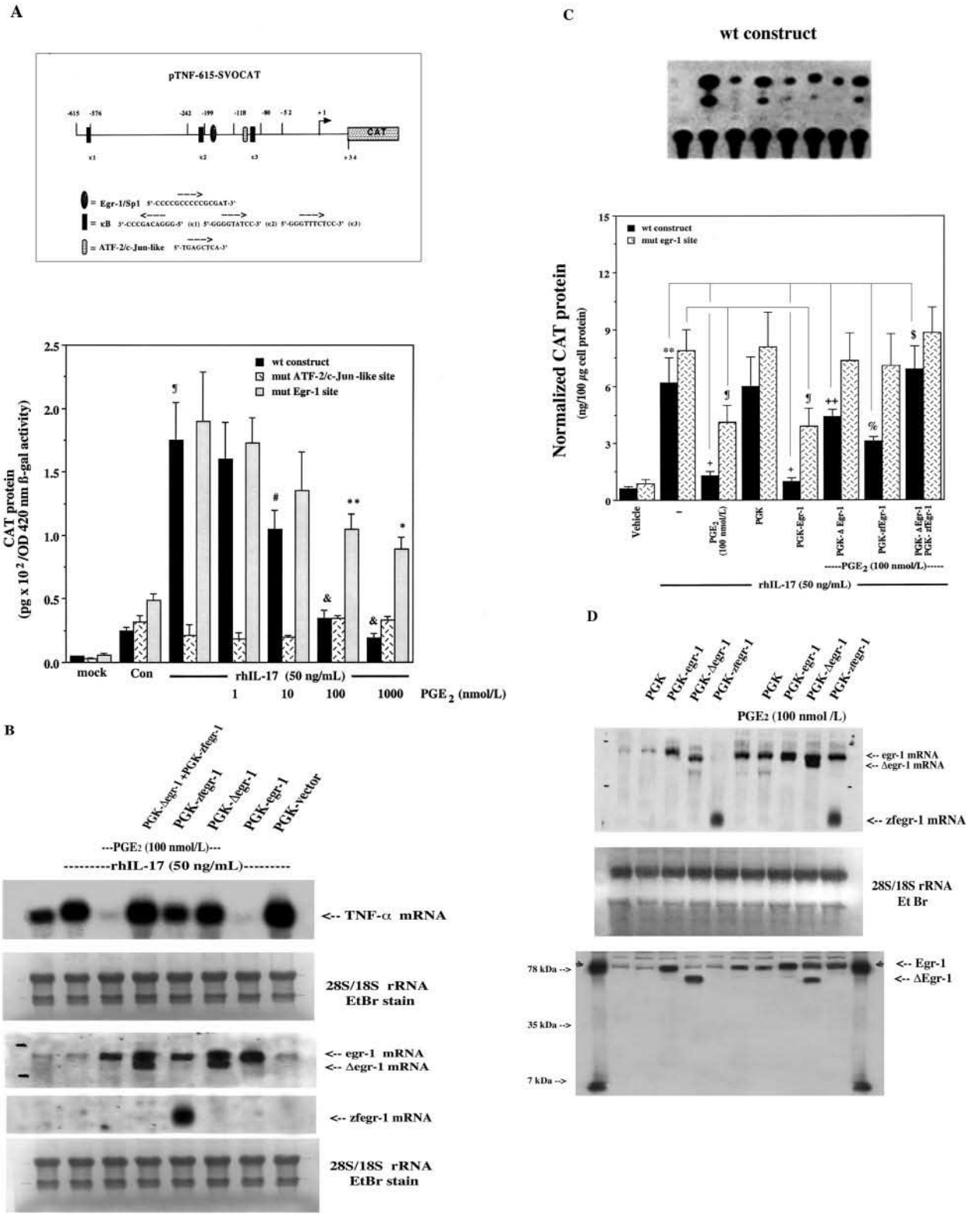

FIG. 5. **Transcriptional repression of rhIL-17-induced TNF-α gene expression by PGE_2: role of Egr-1.** In *A*, THP-1 cells (5×10^7 cells/0.5 ml), cultured in RPMI 1640 medium, 10% FCS, and antibiotics, were transfected by electroporation with 10 µg of total WT, mutant (*mut*) Egr-1, and mutant ATF-2/c-Jun pTNF−615SVOCAT plasmids using a 7-s pulse at 300 V and 960 microfarads. Transfection efficiencies were controlled in all experiments by cotransfection with 0.5 µg of pCMV-β-gal construct. Cells were allowed to recover for 24 h in complete medium, after which they were maintained under low serum conditions (1% for 3 h) and then treated with or without rhIL-17 (50 ng/ml) in the absence or presence of increasing concentrations of PGE_2 for 6 h. Cells were washed and lysed, and promoter activities were assessed by measuring CAT protein using a specific and sensitive enzyme-linked immunosorbent assay. β-Galactosidase (*β-gal*) activity was measured as described under "Materials and Methods." *Con*, control. In *B*, THP-1 cells (10^7 cells/0.5 ml) or THP-1 cells stably transfected with a eukaryotic expression vector (PGK vector, PGK-Egr-1, PGK-*zf*Egr-1, or PGK-ΔEgr-1) were incubated with or without rhIL-17 (50 ng/ml) in the presence or absence of PGE_2 (100 nmol/liter) for 4 h, after which the cells were extracted for total RNA, and 5 µg were analyzed for TNF-α and *egr-1* mRNAs by Northern hybridization using specific digoxigenin-labeled cDNA probes as described under "Materials and Methods." In *C*, NIH-3T3 cells (10^6 cells/well) stably transfected with a eukaryotic expression vector (PGK vector, PGK-Egr-1, PGK-*zf*Egr-1, or PGK-ΔEgr-1) were transiently cotransfected with 10 µg of WT or mutant Egr-1 site pTNF−615SVOCAT plasmid using Lipofectamine™ 2000 for 6 h according to the manufacturer's protocol with

pletely abrogate PGE_2-dependent inhibition of induced promoter activity (Fig. 5*A*).

To better characterize the putative role of Egr-1 in mediating direct PGE_2 inhibition of TNF-α expression, we chose to use THP-1 cells stably transfected with eukaryotic expression constructs of WT Egr-1 and dominant-negative mutants (see "Materials and Methods"). As shown in Fig. 5*B*, the suppressive effects of PGE_2 on TNF-α mRNA expression were reversed significantly by the dominant-negative mutants PGK-ΔEgr-1 and PGK-*zf*Egr-1, and the blockade was essentially complete when both were transfected in tandem. Moreover, high level expression of WT PGK-Egr-1 in THP-1 cells inhibited rhIL-17-induced TNF-α mRNA expression as efficiently as did PGE_2 (Fig. 5*B*).

Because the latter experiments did not rule out the possibility that overexpressed Egr-1 constructs affect TNF-α mRNA stability (as distinct from the transcriptional control), we chose to repeat these experiments with stable NIH-3T3 transformants expressing Egr-1 (PGK-Egr-1) and the dominant-negative mutants PGK-ΔEgr-1 and/or PGK-*zf*Egr-1, in which, however, the TNF-α promoter construct was transfected transiently. Our aim was to use a cell culture model that does not express TNF-α mRNA normally (eliminating low signal-to-noise ratios) and in which substantial transient transfection efficacy can be attained. PGK-Egr-1 reduced rhIL-17-stimulated WT pTNF-SVOCAT activity to near vehicle levels, mimicking to a large degree the effects of PGE_2 in this regard (Fig. 5*C*). The dominant-negative mutants PGK-ΔEgr-1 and PGK-*zf*Egr-1, alone or in combination, substantially reversed the inhibitory effects of exogenously added PGE_2. Both CAT protein and CAT activity measurements gave very similar results. However, as in the data shown in Fig. 5*A*, mutating the Egr-1 site did not completely reverse PGE_2/Egr-1-dependent inhibition of rhIL-17-induced promoter activity. As shown in Fig. 5*D*, the level of expression of all the Egr-1 and mutant constructs was quite substantial (and adequate) compared with endogenous expression of Egr-1 in untreated cells and was represented essentially stoichiometrically in cells treated with PGE_2.

Thus, the previous results presented us with a conundrum: on the one hand, the data provided strong evidence that Egr-1 mediated the inhibitory effects of PGE_2 on induced TNF-α mRNA expression, whereas on the other, PGE_2-stimulated Egr-1 binding to its cognate site in the TNF-α promoter was not wholly adequate for complete (induced) promoter suppression. We thus explored the possibility that PGE_2/Egr-1 may disrupt the rhIL-17-stimulated TNF-α transcriptional enhancer complex. In preliminary experiments using NIH-3T3 cells overexpressing "decoy" constructs (see Ref. 33 using a human synovial fibroblast cell strain) that harbor the transactivation domains of ATF-2, c-Jun, CREB-1, Elk-1, and c-Fos fused to the Gal4 DNA-binding domain, we observed that rhIL-17 induction of the TNF-α promoter CAT activity was blocked by pSV40-Gal4-ATF-2-(1–96) and pSV40-Gal4-c-Jun-(1–223) (compare 0.71 ± 0.22 (control) and 6.3 ± 0.79 (rhIL-17) normalized CAT protein at ng/μg of cellular protein *versus* 0.65 ± 0.19 (ATF-2; $p < 0.0002$); 3.1 ± 0.75 (c-Jun; $p < 0.043$), 5.4 ± 1.09 (CREB-1), 6.6 ± 1.2 (c-Fos), and 6.1 ± 2.1 (Elk-1)). Gel shift experiments were undertaken to substantiate the latter findings; and as shown in Fig. 6*A*, rhIL-17 increased the binding to an ATF-2/c-Jun-like ^{32}P-labeled oligonucleotide of the human TNF-α promoter. Substantial supershifted low mobility banding was observed with anti-phospho-ATF-2 ($Thr^{69/71}$) and anti-phospho-c-Jun ($Ser^{63/73}$) antibodies, but not with anti-phospho-CREB-1 (Ser^{133}) antibody (Fig. 6*A*, *upper panel*). The addition of PGE_2 and overexpression of PGK-Egr-1 blocked rhIL-17-stimulated binding to the ATF-2/c-Jun-like ^{32}P-labeled oligonucleotide to a substantial degree (Fig. 6*A*, *lower left panel*). The inhibitory effect of PGE_2 was markedly abrogated in cells stably transfected with the dominant-negative mutants PGK-ΔEgr-1 and PGK-*zf*Egr-1, alone or in combination (Fig. 6*A*, *lower right panel*).

We next examined the possible role of Egr-1 in the mediation of PGE_2 effects on c-Jun (AP-1) using NIH-3T3 transformants expressing Egr-1 (PGK-Egr-1) and the dominant-negative mutants PGK-ΔEgr-1 and PGK-*zf*Egr-1, alone or in combination. As shown in Fig. 6*B*, rhIL-17-induced c-*jun* mRNA expression was suppressed by PGE_2, an effect mimicked by Egr-1, but compromised by the Egr-1 dominant-negative mutants. In tandem comparative studies, *junB*, a wholly inducible gene and potent transcriptional repressor of c-Jun transactivation activity (37), was not responsive to rhIL-17 treatments, but was up-regulated by Egr-1 overexpression. Furthermore, PGE_2 markedly up-regulated the steady-state expression of *junB* mRNA, and the effect was reversed by the Egr-1 dominant-negative mutants. JunB was not detectable in gel shift experiments (data not shown).

DISCUSSION

There is now wide agreement that TNF-α is a key cytokine mediator in the immune and inflammatory responses, initiating a cascade of processes that ultimately serve to eliminate the invading pathogen and to re-establish a state of homeostasis in the organism (4, 5). However, in chronic and systemic inflammatory states, such as those seen in RA, excess TNF-α production is believed to be associated with disease pathogenesis, and remedial results have been reported with disease-modifying anti-TNF-α therapies (38). Somewhat paradoxically, we recently reported that TNF-α production by synovial membrane/pannus explants from osteoarthritis/RA patients was very modest, but could be increased markedly by treating the explants with preferential COX-2 inhibitors, a process reversed by exogenously added PGE_2 (36). Furthermore, LPS-stimulated release of TNF-α was increased 4–5-fold in the presence of the COX-2 inhibitors, a response strongly reversed by exogenous PGE_2. Given the clinically relevant nature of the studies, we concluded that TNF-α expression is largely eicosanoid-dependent. Thus, the present work is a logical extension of our previous studies (16, 23, 36) and delves into the complex and novel mechanistic details of PGE_2 regulation of TNF-α synthesis.

The production of TNF-α is controlled transcriptionally, post-transcriptionally (mRNA stability), and translationally and by post-translational processing (TNF-α-converting enzyme), although the precise mechanisms involved remain a challenge for

cells at 30–40% confluence. Cells were then re-exposed to culture medium and 1% FCS for 2 h to ensure quiescence and synchrony prior to the addition of rhIL-17 (50 ng/ml) with or without PGE_2 (100 nmol/liter) as indicated, and cells were extracted after 16 h for analysis of CAT protein and activity as described under "Materials and Methods." In *D*, NIH-3T3 cells stably transfected with the PGK vector, PGK-Egr-1, PGK-*zf*Egr-1, or PGK-ΔEgr-1 were extracted for total RNA; 5 μg were analyzed for *egr-1/krox-24* mRNA by Northern hybridization using a specific digoxigenin-labeled cDNA probe as described under "Materials and Methods," and 50 μg of protein were analyzed for Egr-1/Krox-24 protein by Western blotting using a specific polyclonal antiserum to Egr-1 (purified Egr-1 protein; *first* and *last lanes*). Probabilities were calculated by Student's *t* test. *B*: ¶, $p < 0.0001$ *versus* control WT construct; #, $p < 0.04$ *versus* rhIL-17 WT construct; &, $p < 0.001$ *versus* rhIL-17 WT construct. *C*: **, $p < 0.0027$ *versus* vehicle; +, $p < 0.005$ *versus* rhIL-17; ¶, $p < 0.043$ *versus* rhIL-17 with mutant Egr-1; ++, $p < 0.01$ *versus* rhIL-17 with PGE_2; %, $p < 0.027$ *versus* rhIL-17 with PGE_2; $, $p < 0.0025$ *versus* rhIL-17 with PGE_2.

future research. Here, we have reported that the T-cell-derived pro-inflammatory cytokine IL-17 up-regulated TNF-α expression and synthesis in human macrophages and RASFs largely through ATF-2/c-Jun-dependent transactivation at a 5′-TGAGCTCA-3′ palindrome of the TNF-α promoter. In previous reports, IL-17 stimulated ATF-2/CREB-1-dependent transactivation of the COX-2 promoter at a 5′-TTTCGTCA-3′ site, and the cytokine cell signaling profile was also restricted to the p38 MAPK cascade in a number of different fibroblast and mesenchymal phenotypes (33). As an example of the complexity and subtlety of cell signaling, the PGE_2-targeted site of the *egr-1* promoter (5′-TCACGTCA-3′) binds preferentially, although not exclusively, ATF-2 dimers, the latter phosphorylated by p38 MAPK, arguing that signaling intermediates may be "selected" at the level of the promoter sequence.

Several studies suggest that IL-17 signals through NF-κB, and this may be of some significance because the TNF-α promoter has three putative NF-κB-like binding sites (39, 40). However, in all cell types we are concerned with, IL-17 activation of NF-κB is modest, delayed, or inhibited, whereas others have shown that these putative enhancer sites do not mediate the induction of the human TNF-α promoter by substances known to activate NF-κB (*i.e.* LPS) (see Refs. 32, 40, and 41). In support of this, it was recently shown that CREB-binding protein/p300 mediates LPS induction of TNF-α through assembly of an enhancer complex that includes Ets-1/Elk-1 binding to an NF-AT site, ATF-2/c-Jun dimer binding to the ATF-2/CRE site, and GC box-binding transcription factors in murine monocytic J774/P338D cells (41). It would be of interest, however, to determine whether the latter response is species-specific, as LPS signals through Toll-like receptor-4 in human macrophages and activates the NF-κB cascade (42). Interestingly, TNF-α controls it own synthesis (autoregulation), and a putative palindrome that resembles an ATF/CRE site (bp −125 to −82) apparently mediates TNF-α-dependent promoter induction (43); the transactivation factors were identified as exclusively c-Jun-related proteins. The observed discrepancies may be the result of cell context, and it seems likely that a gene can respond to multiple signaling cascades through the recruitment of different proteins to similar enhancer elements. It should also be mentioned that there are data demonstrating ATF-2/c-Jun transactivation at AP-1 sites of certain promoters as well (44).

As a transcription factor, Egr-1 induces the promoter activity of prostaglandin E synthase directly by binding to tandem GC boxes in the proximal promoter (10), but can act in combinatorial fashion with other transcription factors, as in the case of its own expression in granulosa cells subsequent to follicle-stimulating hormone and luteinizing hormone stimulation (46). In the latter system, Egr-1 associates with Sp-1 to bind a proximal GC box and together with an enhancer complex involving CREB and serum response factor to drive *egr-1* mRNA expression. This paradigm is not well suited to our results, as PGE_2 stimulation of Egr-1 protein was delayed by ~30 min, whereas PGE_2-dependent transcriptional induction of *egr-1* mRNA was rapid. Our data favor the rapidly induced p38 MAPK pathway with ATF-2/CREB transactivation of the *egr-1* promoter at cognate sites, although the increase in *egr-1* mRNA seen at 4 h may be Egr-1-driven. In stromal cells, up-regulated macrophage colony-stimulating factor gene expression by TNF-α or IL-1β involves Sp-1-dependent promoter transactivation, and this occurs subsequent to Egr-1 phosphorylation and dissociation from Sp-1, resulting in higher levels of free, transcriptionally active Sp-1 (47). In contrast, Sp-1 and Egr-1 have opposing effects on the regulation of the multidrug resistance gene *MDR1*, where the latter transcription factor suppresses *MDR1*

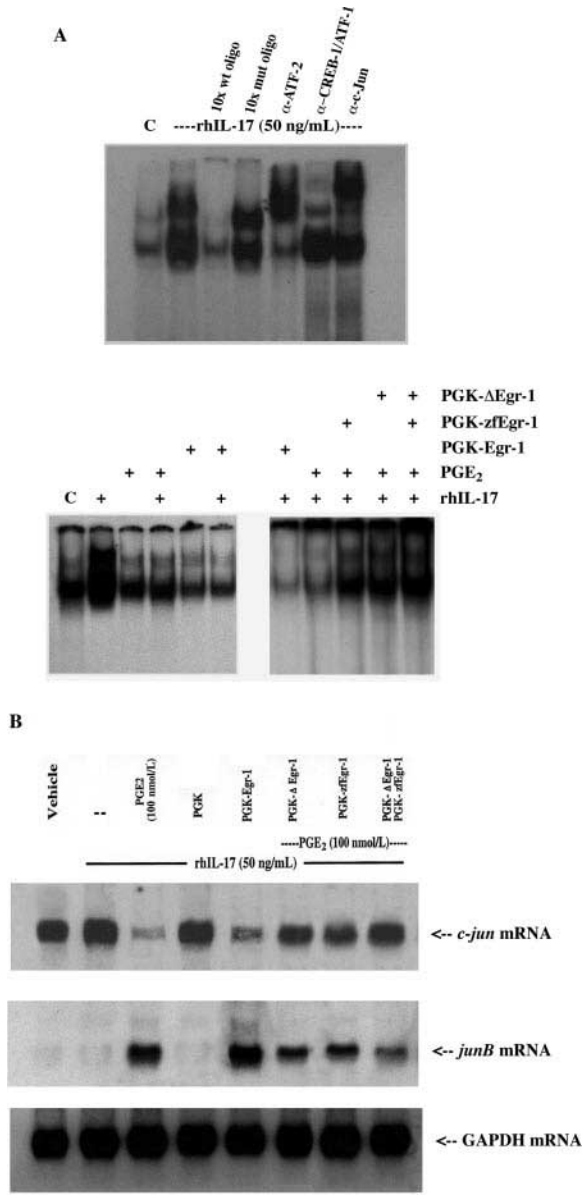

FIG. 6. **Disruption of rhIL-17-induced binding to a TNF-α promoter-specific ATF-2/c-Jun ^{32}P-labeled oligonucleotide by PGE_2: role of Egr-1-mediated suppression of c-*jun* mRNA expression.** In *A*, quiescent RASFs were treated with rhIL-17 or vehicle (control (*C*)) for 60 min, after which nuclear proteins were extracted, and EMSA and supershift analyses (using an ATF-2/c-Jun sequence oligonucleotide probe and the indicated antibodies) were performed as described under "Materials and Methods" (*upper panel*). Quiescent NIH-3T3 cells (10^6 cells/well) or cells stably transfected with a eukaryotic expression vector (PGK-Egr-1, PGK-*zf*Egr-1, or PGK-ΔEgr-1) were treated with vehicle with or without rhIL-17 (50 ng/ml) in the presence or absence of PGE_2 (100 nmol/liter) for 60 min as indicated (*lower panels*). Nuclear extracts were prepared, and EMSA was performed. *mut*, mutant. In *B*, quiescent NIH-3T3 cells (10^6 cells/well) or cells stably transfected with a eukaryotic expression vector (PGK vector, PGK-Egr-1, PGK-*zf*Egr-1, or PGK-ΔEgr-1) were treated with vehicle or with rhIL-17 in the presence or absence of PGE_2 for 30 min as indicated. Cells were extracted for total RNA, and 5 μg were analyzed for c-*jun*, *junB*, and GAPDH mRNAs by Northern hybridization using specific digoxigenin-labeled cDNA probes as described under "Materials and Methods."

promoter activity, and the former activates it (48). Sp-1 expression in our cell culture models is constitutive, and its binding to GC-rich sequences (Egr-1 or TNF-α promoters presumably) appears to be unchanged under the treatments we used. We can not rule out the possibility, however, that Sp-1 transactivation activity may be modified by phosphorylation/glycosyla-

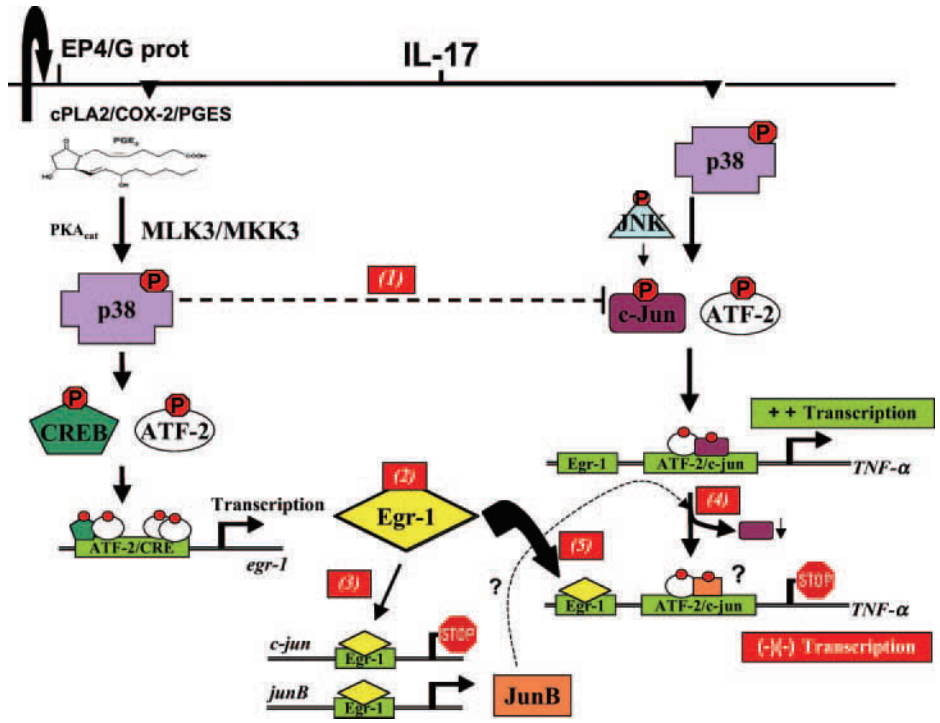

FIG. 7. **Proposed mechanism of PGE_2-dependent suppression of IL-17-induced TNF-α synthesis in human macrophages.** In human RA-affected synovial membranes, CD45 T-lymphocyte-derived IL-17 activates a resident macrophage/synovial cell to produce TNF-α through strong ATF-2/c-Jun-dependent promoter transactivation (rapid response). In tandem, the cytosolic phospholipase A_2 (*cPLA2*)/COX-2/prostaglandin E synthase (*PGES*) cascade is stimulated, with the resultant production of PGE_2 (delayed). Through an EP4 receptor-mediated negative feedback loop, PGE_2 activates the MLK3/MKK3/p38 MAPK and protein kinase A signaling pathways. This results, more or less simultaneously, in the possible inhibition of c-Jun phosphorylation (*1*), stimulation of Egr-1 synthesis through ATF-2/ATF-2:ATF-2/CREB-dependent promoter transactivation (*2*), and Egr-1 inhibition of c-*jun* mRNA expression (*3*) likely through promoter suppression and disruption of the IL-17-induced ATF-2·c-Jun enhancer complex (*4*). The mechanism of Egr-1 binding and partial TNF-α promoter suppression remains to be determined (*5*). *Thick arrows* indicate strong experimental support, whereas *broken lines/thin arrows* indicate putative mechanisms. The proposed mechanism is consistent with our previous observations (36) regarding the autocrine/paracrine regulation of TNF-α production by endogenously produced PGE_2. *G prot*, G-protein.

tion subsequent to PGE_2 stimulation (or Egr-1 overexpression).

A previous study reported that transient Egr-1/Krox-24 overexpression in Jurkat T-cells could increase TNF-α promoter activity, although deletion mutants failed to ascribe the inductive effect to the Egr-1 site in the promoter (29). Given the problems inherent in transient overexpression with cotransfected promoter constructs (*e.g.* low efficiency), we chose the more laborious route of stable transfectants with the goal of obtaining consistent results. Well tested procedures (28, 49) were used to ensure amplification of those clones in which *egr-1* mRNA expression was substantial and Egr-1 protein was obtained while at the same time not inducing background expression as a result of transfection. The PGK-ΔEgr-1/Krox-24 mutant has been shown to interact with endogenous Egr-1 and to reduce its transactivation capacity in reporter studies, whereas the PGK-*zf*Egr-1/Krox-24 mutant probably saturates cognate enhancer-binding sites (28, 49). The culture system afforded us strong evidence that PGE_2 suppression of induced TNF-α mRNA expression occurs through a transcriptional mechanism involving the Egr-1-mediated inhibition of c-Jun expression and disruption of the IL-17-activated transactivation complex (Fig. 7). The concomitant induction of JunB, a transcriptional repressor (50), became evident on a tandem mRNA screen and is likely mediated by an Egr-1 site in the proximal promoter region (51). Whether JunB, which binds DNA very weakly (50), competes with c-Jun for ATF-2 dimerization and thus causes DNA binding displacement is under further study.

Recent studies suggest that PGE_2, via the EP4 receptor, induces *egr-1* expression via activation of phosphatidylinositol 3-kinase, ERK1/2, and glycogen synthase kinase-3 (24). Downstream targets of this activation include cyclin D and T-cell factor (52). Considering the data presented here and our previous reports (16, 21), PGE_2 seems to induce Egr-1 synthesis by rapid activation of the MLK3/MKK3/SAPK2αβ/p38 MAPK/MSK1 cascade with strong ATF-2/ATF-2:ATF-2/CREB-1 transactivation of the target promoter. However, PGE_2 also stimulates MEK1/2 and ERK1/2 phosphorylation, but is delayed by 1 h in our cell culture models (16), and preliminary results suggest that the eicosanoid drives ERK1/2 ubiquitination and proteasomal processing, as if to terminate an extraneous signal. We propose that PGE_2, acting via feedback or feed-forward mechanisms, interdicts the pro-inflammatory response of cytokines by shutting down cytokine-activated cascades, *i.e.* p38 MAPK via MKK3 inhibits JNK1 phosphorylation and AP-1 activity and turns off c-Jun-targeted genes. The latter scenario may represent one of many cellular pathways ensuring homeostasis: differentiation and developmental signals counterbalancing transformational/oncogenic cues.

Whether Egr-1 has a role to play in the acute or chronic inflammatory response remains a subject for further research; however, a number of observations may be informative. The RA-affected synovial membrane/pannus expresses markedly elevated levels of *egr-1* mRNA (53),[2] whereas other oncogenes such as c-*jun* and c-*fos*, for example, are not detectable. COX-2 protein and ambient PGE_2 levels are extremely high, whereas pro-inflammatory cytokine production is very low until, as mentioned above, preferential COX-2 inhibitors suppress PGE_2 production (36). Egr-1-null mice have compromised pituitary-adrenal axis function (45), presumably leading to a suboptimal

[2] J. A. Di Battista, unpublished data.

endogenous anti-inflammatory response. Thus, PGE_2/Egr-1 may form a critical axis involved in the modulation of the immune (27) and inflammatory responses and may be essential for the programming of cellular differentiation.

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REFERENCES

1. Luster, A. D. (1998) *N. Engl. J. Med.* **338,** 436–445
2. Hasunuma, T., Kato, T., Kobata, T., and Nishioka, K. (1998) *Springer Semin. Immunopathol.* **20,** 41–52
3. Burg, N. D., and Pillinger, M. H. (2001) *Clin. Immunol.* **99,** 7–17
4. Lotz, M. (1997) in *Arthritis and Allied Conditions* (Koopman, W. J., ed) 13th Ed., pp. 439–478, Williams & Wilkins, Baltimore
5. Harris, S. G., Padilla, J., Koumas, L., Ray, D., and Phipps, R. P. (2002) *Trends Immunol.* **23,** 144–150
6. Funk, C. D. (2001) *Science* **294,** 1871–1875
7. Smith, W. L., Borgeat, P., Fitzpatrick, F. A. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J., eds) pp. 297–325, Elsevier Science Inc., New York
8. Evans, J. H., Spencer, D. M., Zweifach, A., and Leslie, C. C. (2001) *J. Biol. Chem.* **276,** 30150–30160
9. Smith, W. L., Dewitt, D. L., and Garavito, M. (2000) *Annu. Rev. Biochem.* **69,** 145–182
10. Naraba, H., Yokoyama, C., Tago, N., Murakami, M., Kudo, I., Fueki, M., Oh-ishi, S., and Tanabe, T. (2002) *J. Biol. Chem.* **277,** 28601–28608
11. Narumiya, S., and Fitzgerald, G. A. (2001) *J. Clin. Investig.* **108,** 25–30
12. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) *Physiol. Rev.* **79,** 1193–1226
13. Sugimoto, Y., Narumiya, S., and Ichikawa, A. (2000) *Prog. Lipid Res.* **39,** 289–314
14. He, Q. W., He, Y., Faour, W., and Di Battista, J. A. (2001) *Osteoarthritis Cartilage* **9,** (suppl.) S9
15. Morin, N., Kiansa, K., Martel-Pelletier, J., Pelletier, J.-P., and Di Battista, J. A. (1999) *Osteoarthritis Cartilage* **7,** (suppl.) S21
16. Faour, W., He, Y., and Di Battista, J. A. (2000) *Osteoarthritis Cartilage* **8,** (suppl.) S10
17. McCarthy, T. L., Casinghino, S., Mittanck, D. W., Ji, C.-H., Centrella, M., and Rotwein, P. (1996) *J. Biol. Chem.* **271,** 6666–6671
18. Austin, S. C., and Funk, C. D. (1999) *Prostaglandins Other Lipid Mediat.* **58,** 231–252
19. William, C. S., Mann, M., and DuBois, R. N. (1999) *Oncogene* **18,** 7908–7916
20. Marinissen, M. J., and Gutkind, J. S. (2001) *Trends Pharmacol.* **22,** 368–376
21. Faour, W. H., He, Y., de Ladurantaye, M., He, Q. W., Quintero, M., Mancini, A., and Di Battista, J. A. (2001) *J. Biol. Chem.* **276,** 31720–31731Q. W.
22. Fang, M. A., Noguchi, G. M., and McDougall, S. (1996) *Prostaglandins Leukotrienes Essent. Fatty Acids* **54,** 109–114
23. Di Battista, J. A., Martel-Pelletier, J., and Pelletier, J.-P. (1999) *Osteoarthritis Cartilage* **7,** 395–398
24. Fujino, H., Xu, W., and Regan, J. W. (2003) *J. Biol. Chem.* **278,** 12151–12156
25. Gashler, A., and Sukhatme, V. P. (1995) *Prog. Nucleic Acid Res. Mol. Biol.* **50,** 191–223
26. Khachigian, L. M., and Collins, T. J. (1998) *Mol. Med.* **76,** 613–616
27. Nguyen, H. Q., Hoffmann-Liebermann, B., and Liebermann, D. A. (1993) *Cell* **72,** 197–209
28. Lanoix, J., Mullick, A., He, Y., Bravo, R., and Skup, D. (1999) *Oncogene* **17,** 2496–2504
29. Kramer, B., Meichle, A., Hensel, G., Charnay, P., and Kronke, M. (1994) *Biochim. Biophys. Acta* **1219,** 413–421
30. Khachigian, L. M., Lindner, V., Williams, A. J., and Collins, T. (1996) *Science* **271,** 1427–1431
31. Khachigian, L. M., Williams, A. J., and Collins, T. (1995) *J. Biol. Chem.* **270,** 27679–27686
32. Jovanovic, D. V., Di Battista, J. A., Martel-Pelletier, J., Jolicoeur, F. C., He, Y., Zhang, M. K., Mineau, F., and Pelletier, J.-P. (1998) *J. Immunol.* **160,** 3513–3521
33. Faour, W. H, He, Q. W., Mancini, A., and Di Battista, J. A. (2003) *J. Biol. Chem.* **278,** 26897–26907
34. Svetlov, A., and Nigami, S. (1993) *Biochim. Biophys. Acta* **1177,** 75–79
35. Aggarwal, B. B., and Puri, R. K. (1995) *Human Cytokines: Their Role in Disease and Therapy*, Blackwell Science Inc., Cambridge, MA
36. He, Q. W., Pelletier, J.-P., Martel-Pelletier, J., Laufer S., and Di Battista, J. A. (2002) *J. Rheumatol.* **29,** 546–553
37. Jonk, L. J. C., Itoh, S., Heldin, C.-H., ten Dijke, D., and Kruijer, W. (1998) *J. Biol. Chem.* **273,** 21145–21152
38. Harriman, G., Harper, L. K., and Schaible, T. (1999) *Ann. Rheum. Dis.* **58,** 161–167
39. Schwander, R., Yamaguchi, K., and Cao, Z. (2000) *J. Exp. Med.* **191,** 1233–1240
40. Goldfeld, A. E., Doyle, C., and Maniatis, T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9769–9773
41. Tsai, E. Y., Falva, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000) *Mol. Cell. Biol.* **20,** 6084–6094
42. Muzio, M., Poletarutti, N., Bosisio, D., Manoj Kumar, P. P., and Mantovani, A. (2000) *Biochem. Soc. Trans.* **28,** 563–566
43. Leitman, D. C., Ribeiro, R. C., Mackow, E. R., Baxter, J. D., and West, B. L. (1991) *J. Biol. Chem.* **266,** 9343–9346
44. Kawasaki, H., Song, J., Eckner, R., Ugal, H., Chiu, R., Taira, K., Shi, Y., Jones, N., and Yokoyama, K. K. (1998) *Genes Dev.* **12,** 233–245
45. Topilko, P., Schehdermanoury, S., Levi, G., Trembleau, A., Gourdji, D., Priancourt, M. M., Rao, C. V., and Charnay, P (1998) *Mol. Endocrinol.* **12,** 107–122
46. Russell, D. L., Doyle, K. M., Gonzales-Robayna, I., Pipaon, C., and Richards, J. S. (2003) *Mol. Endocrinol.* **17,** 520–533K. M. H.
47. Srivastava, S., Weitzmann, M. N., Kimble, R. B., Rizzo, M., Zahner, M., Milbrandt, J., Ross, F. P., and Pacifici, R. (1998) *J. Clin. Investig.* **102,** 1850–1859
48. Thottassery, J. V., Sun, D., Zambetti, G. P., Troutman, A., Sukhatmer, V. P., Schuetz, E. G., and Schuetz, J. D. (1999) *J. Biol. Chem.* **274,** 3199–3206
49. Lanoix, J., Belhumeur, P., Lussier, M., Royal, A., Bravo, R., and Skup, D. (1991) *Cell Growth & Differ.* **2,** 391–399
50. Chiu, R., and Karin, M. (1989) *Cell* **59,** 979–986
51. De Groot, R. P., Auwerx, J., Karperien, M., Staels, B., and Kruijer, W. (1991) *Nucleic Acids Res.* **19,** 775–781
52. Fujino, H., West, K., and Regan, J. W. (2002) *J. Biol. Chem.* **277,** 2614–2619
53. Trabandt, A., Aicher, W. K., Gay, R. E., Sukhatme, V. P., Fassbender, H. G., Birkedal-Hansen, H., and Gay, S. (1992) *Rheumatol. Int.* **12,** 53–59