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Prostaglandin E_2 Stimulates p53 Transactivational Activity through Specific Serine 15 Phosphorylation in Human Synovial Fibroblasts

*ROLE IN SUPPRESSION OF c/EBP/NF-κB-MEDIATED MEKK1-INDUCED MMP-1 EXPRESSION**

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Cyclooxygenase-2 (COX-2) overexpression has been linked to cell survival, transformation, and hyperproliferation. We examined the regulation of the tumor suppressor gene p53 and p53 target genes by prostaglandin E_2 (PGE_2) in human synovial fibroblasts (HSF). PGE_2 induced a time-dependent increase in p53 Ser^{15} phosphorylation, with no discernible change in overall p53 levels. PGE_2-dependent Ser^{15} phosphorylation was apparently mediated by activated p38 MAP kinase as SB202190, a p38 kinase inhibitor, blocked the response. Overexpression of a MKK3 construct, but not MKK1, stimulated SB202190-sensitive p53 Ser^{15} phosphorylation. PGE_2-stimulated [phospho-Ser^{15}]p53 transactivated a p53 response element (GADD45)-luciferase reporter in transiently transfected HSF (SN7); the effect was compromised by overexpression of a dominant-negative mutant (dnm) of p53 or excess p53S15A expression plasmid but mimicked by a constitutively active p53S15E expression construct. PGE_2, wtp53 expression in the presence of PGE_2, and p53S15E suppressed steady-state levels of MEKK1-induced MMP-1 mRNA, effects nullified with co-transfection of p53 dnm or p53S15A. MEKK1-induced MMP-1 promoter-driven luciferase activity was largely dependent on a c/EBPβ-NF-κB-like enhancer site at −2008 to −1972 bp, as judged by deletion and point mutation analyses. PGE_2, overexpression of p53wt with PGE_2, or p53S15E abolished the MEKK1-induced MMP-1 promoter luciferase activity. Gel-shift/super gel-shift analyses identified c/EBPβ dimers and c/EBPβ/NF-κB p65 heterodimers as binding species at the apparent site of MEKK1-dependent transactivation. PGE_2-stimulated [phospho-Ser^{15}]p53 abrogated the DNA binding of c/EBPβ dimers and c/EBPβ/NF-κB p65 heterodimers. Our data suggest that COX-2 prostaglandins may be implicated in p53 function and p53 target gene expression.

In response to distinct forms of cellular stress, the tumor suppressor gene product p53 becomes activated and functions to maintain cellular and tissular homeostasis by protecting genomic integrity. The latter is largely accomplished through the promotion of cell cycle checkpoints and/or activation of programmed cell death (apoptosis) (1). Inactivating mutants of p53 represent the most frequent genetic alterations in human cancers (2) and account, in large measure, for the continued proliferation of structurally and genetically compromised cells (2–4). The p53 tumor suppressor gene codes for a transcription factor that normally transactivates as a homotetramer recognizing two copies of a 10-bp DNA sequence motif, 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, the latter sequences separated by 0–13 bp. A list of p53 target genes includes PUMA, Bid, p21, GADD45, and Apaf-1, each harboring one or more p53 sites in their promoter regions (5–9). The stabilization and activation of p53 is regulated by protein-protein interactions (*e.g.* with MDM2 ubiquitin ligase) (10) and through multiple post-translational covalent modifications including phosphorylation, acetylation, and sumolation, occurring largely in the N- and C-terminal regions of the protein. For example, CBP/p300-dependent acetylation of Lys^{383} of p53 is required for the transcriptional induction of p21 subsequent to cellular DNA damage (genotoxicity) (11). Phosphorylation of Ser^{33} and Ser^{46} of p53 occurs in a number of cell lines (*e.g.* HeLa, A549, MCF-7) following UV irradiation and results from the activation of the Ras/MAPK[3]/SAPK kinase cascade (12). Inhibition of p38 MAP kinase or mutating Ser^{33} and Ser^{46} to alanine abrogated UV-induced p53 transcriptional activity and p53-dependent apoptosis (12). Other potential MAPK-dependent phosphorylation sites that may modify p53 activity include $Ser^{6,9,15,20,37,46}$,

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; CAT, chloramphenicol acetyl transferase; c/EBP/NF-IL6, CCAAT-binding protein; DMEM, Dulbecco's modified Eagles medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GADD45, growth arrest and DNA damage protein 45; HSF, human synovial fibroblasts; LUC, Luciferase; MEKK1, MAPK/Erk kinase kinase; MEK3/MKK3, MAPK/ERK kinase 3; MEK3/MKK1, MAPK/Erk kinase 1; MMP-1, matrix metalloprotease-1/interstitial collagenase-1; NF-κBp65, nuclear factor κBp65; PGE_2, prostaglandin E_2; rhIL-1β, recombinant human interleukin-1β; SN7, normal fetal human synovial fibroblast cell strain 7; wt, wild type; RLU, relative light units; TNF, tumor necrosis factor.

and Ser^{392} (reviewed in Ref. 13). Interestingly, Ser^{15} and Ser^{37} phosphorylation also occurs following UV exposure in transformed cell lines and is contingent on efficient Ser^{33} and Ser^{46} phosphorylation, suggesting that extensive N-terminal phosphorylation of p53 may be necessary for full activity (12, 14).

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder, with systemic features and joint involvement, resulting in erosive synovitis with hyperproliferation of synovial lining cells, cartilage matrix degradation, and joint destruction (15). The underlying molecular basis of matrix degradation is thought to be dependent on the action of a number of proteolytic enzymes (matrix metalloprotease, MMPs) that may be produced by both soft and hard tissue elements and by inflammatory cells (16). The etiology of synovial hyperproliferation is ill defined with anecdotal reports of elevated/aberrant oncogene, growth factor and adhesion molecule synthesis (reviewed in Ref. 15). Firestein *et al.* (17) reported that RA lining cells expressed elevated levels of p53 and that mutant p53 transcripts, identical to those isolated from human tumors, are also present in a subset of RA synovium (18). The mechanism sustaining p53 expression remains unclear although it is hypothesized that a putative genotoxic environment in RA joints may cause DNA strand breaks.

A role for cyclooxygenase-2 (COX-2) in neoplasmic transformation has attracted considerable attention particularly in light of the clinical studies indicating that COX-2 inhibitors may have chemotherapeutic value (reviewed in Ref. 19). As yet no consensus has been achieved on mechanistic aspects of the putative chemopreventive effects. We recently demonstrated that elevated and persistent expression of COX-2 in RA-affected synovial membranes may be the result of a controlled positive feedback loop involving prostaglandin E_2 (PGE_2) (20). Ambient levels of PGE_2 are very high in the RA synovial environment (21) and the eicosanoid sustains COX-2 through a prostaglandin E_2 EP4 receptor/$MKK_{3/6}$/p38 MAP kinase/protein kinase A (PKA)-mediated process involving transcriptional but primarily post-transcriptional mechanisms (20). In RA synovial tissue explants, PGE_2-dependent effects account for elevated levels of AP-1 family members (c-Jun excepted) and the potent cell growth stimulating transcription factor, Egr-1 (22, 23). In contrast, the eicosanoid potently suppresses interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) as well as the matrix-destructive metalloprotease MMP-1 to the extent that the latter substances are only detectable when the membranes are treated with nonsteroidal anti-inflammatory drugs (21, 23). Given the plausible link established between p53, MMP-1 overexpression, and RA synovial hyperproliferation (24), we sought to clarify the role COX-2 expression and associated prostaglandin release in synovial pathology by examining the response of p53 and MMP-1 to PGE_2 signaling. In principle, the results could also provide a paradigm for the role of COX in cellular transformation to a cancerous phenotype, tumor invasion, and metastasis. The data presented here show that stress kinase (MEKK1)-induced MMP-1 expression in human arthritis-affected synovial cells was mediated, in large part, by c/EBPβ (NF-κBp65) promoter transactivation. Furthermore, PGE_2-dependent suppression of MEKK1-induced MMP-1 expression occurred, in large measure, through the transcriptional activation of p53, a process dependent on specific Ser^{15} phosphorylation by p38 MAP kinase. We provide evidence that [phospho-Ser^{15}]p53 may physically interact with c/EBPβ or induce proteins that interact with c/EBPβ to reverse MMP-1 promoter activation.

EXPERIMENTAL PROCEDURES

Chemicals—Sodium fluoride, leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride, dithiothreitol, sodium orthovanadate, ethidium bromide, and bovine serum albumin were products of Sigma-Aldrich Canada. Prostaglandin E_2 (PGE_2) and SB202190 were purchased from Calbiochem (La Jolla, CA). SDS, acrylamide, bis-acrylamide, agarose, ammonium persulfate, and Bio-Rad protein reagent originated from Bio-Rad. Tris-base, EDTA, $MgCl_2$, $CaCl_2$, chloroform, dimethylsulfoxide, anhydrous ethanol (95%), methanol (99%), formaldehyde, and formamide were obtained from Fisher Scientific. Dulbecco's modified Eagle medium (DMEM), phosphate-free, and phenol-red-free DMEM, TRIzol reagent, heat-inactivated fetal bovine serum, and an antibiotic mixture (10,000 units of penicillin (base), 10,000 μg of streptomycin (base)) were products of Invitrogen.

Specimens and Cell Culture—Human synovial fibroblasts (HSF) were released from non-diseased and RA/OA articular synovia by sequential enzymatic digestion as described previously (20). Arthritis patients undergoing arthroplasty were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA/RA (mean age 67 $\pm$ 19: F/M 5:1). Cells thus released were transferred to culture dishes for propagation in DMEM, supplemented with 10% FCS and antibiotics and were routinely used from the 2nd to the 7th passage so that our cell population was confined largely to type B synovial fibroblasts. Our HSF cell strains were screened for optimal transfection efficiency (40–70%) and three of them SN (synovia normal) 3, SN5, and SN7, isolated from fetal donors at necropsy, were used routinely for stable and transient transfections as previously described (20).

Preparation of Cell Extracts and Western Blotting—50–100 μg of nuclear protein extracted in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 450 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF) or in hot SDS-PAGE loading buffer (total cellular protein), were subjected to SDS-PAGE through 8–12% gels (16 $\times$ 20 cm, final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Biosciences). Western blotting procedures were performed as previously described (20), and membranes were prepared for autoradiography (ECL chemiluminescence reagent, Amersham Biosciences) and subjected to digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, ON, Canada) for semi-quantitative measurements as previously described (20). The following polyclonal antibodies were purchased from Cell Signaling Technology (Waverly MA): Total (independent of phosphorylation state) p53; anti-phospho-p53 (Ser^6, Ser^9, Ser^{15}, Ser^{20}, Ser^{37}, Ser^{46}, Ser^{392}); total and anti-phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}); total MKK3 and MKK1. Anti-human

GADD45 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Northern Blot Analysis of mRNA—Total cellular RNA was isolated (1×10^6 cells = 10–20 μg of RNA) using the TRIzol (Invitrogen) reagent, and 5 μg of total RNA were resolved on 1.2% agarose-formaldehyde gel and transferred electrophoretically (30 V overnight at 4 °C) to Hybond-N™ nylon membranes (Amersham Biosciences) in 0.5× Tris/acetate/EDTA (TAE) buffer, pH 7 (20). After prehybridization for 24 h, hybridizations were carried out at 50 °C for 24–36 h, followed by high stringency washing at 68 °C in 0.1× SSC, 0.1% SDS. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization; human interstitial collagenase (MMP-1) cDNA (1.0 kb, EcoRI-BamHI fragment, kindly provided by Dr. D. R. Edwards, University of Calgary, Alberta, Canada); 780-bp PstI/XbaI fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection). This latter probe served as a control of RNA loading as GAPDH is constitutively expressed. All blots were subjected to a digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada) for semiquantitative measurements.

RT-PCR—2 μg of total RNA, extracted with the TRIzol reagent, were reverse-transcribed and then subjected to PCR with the enzymes and reagents of the GeneAmP RNA PCR kit manufactured by PerkinElmer Cetus (Norwalk, CT) as previously described (20, 26). Both the RT and PCR reactions were done in a Gene ATAQ Controller (Amersham Biosciences). We found a linear range (log luciferase/GAPDH *versus* log cycle number) between 10 and 17; as such we chose 11–13 cycles depending on the type of experiment. The sequences for the luciferase primers were as follows: 5′-ACGGATTACCAGGGATTTCAGTC-3′, and 5′-AGGCTCCTCAGAAACAGCTCTTC-3′ (antisense) for the luciferase fragment of 367 bp (20, 27). The sequences for the GAPDH (which served as a standard of quantitation) primers were 5′-CAGAACATCATCCCTGCCTCT-3′, which corresponds to position 604–624 bp of the published sequence and 5′-GCTTGACAAAGTGGTCGTTGAG-3′, from position 901–922 bp, for an amplified product of 318 bp (26).

Plasmid Constructs, Transfections, and Reporter Assays—The wtpCMV-p53 expression vector and dominant-negative mutant (pCMV-p53mt135) were obtained from Clontech (Palo Alto, CA) and differ only by a conversion of G → A at nucleotide 1017 (amino acid Cys → Tyr). The mutant can form heterotetramers with p53wt but does not bind DNA specifically because of conformational changes in the protein. The p53 amino acid substitution mutants, S15E (Ser → Glu) and S15A (Ser → Ala) were constructed from p53wt expression vector using the QuikChange kit (Stratagene) as previously described (23, 26). The core primer used was as follows (sense): 5′-CTA GCG TCG AGC CCC CTC TG-**GAA(E)** and **GCA(A)**. The p53RE(GADD45)-LUC reporter construct, pCMV-MEK3 (MKK3/6) and pCMV MEK1 (MKK1) expression plasmids were purchased from Stratagene. The latter expression constructs code for full-length rat and human kinases, respectively. The human MMP-1 promoter-LUC construct (−4372–+63) was a generous gift from Dr. Constance Brinckerhoff (Dartmouth Medical School, Hanover, NH). Deletion mutants were developed by restriction endonuclease cleavage and/or by PCR. Point mutations in the region of interest (−2008 to −1972 bp) were generated using specific primers and QuikChange technology. Point mutations at the NF-κB and/or c/EBP-like site were established with the following sense primers: 5′-TGA CGT CTT AGG CAA AAT CCT GTC CAA TCA CAG ATG-3′; 5′-TGA CGT CTT AGG CAA TTT CCT GTC CGG TCA CAG ATG-3′; 5′-TGA CGT CTT AGG CAA AAT CCT GTC C*GG* TCA CAG ATG-3′.

Plasmids containing AP-1 (7×), NF-κB (5×), PPRE (5×), GRE (4×), c/EBP (5×), CRE/ATF-2 (4×), Egr-1 (4×), and ISRE (5×) enhancer elements fused to a basic promoter element (TATA box) and a CAT reporter were purchased from Stratagene or constructed by inserting the tandem enhancer element array in the multiple cloning site of the pFR CAT plasmid (Stratagene). The latter signal transduction pathway reporting plasmids were used in cotransfection experiments with the human MMP-1 promoter LUC reporter system as signaling decoys as previously described (26). A human c/EBPβ eukaryotic expression vector pSCT-LAP was kindly provided by Dr. Lee Wall (University of Montreal, Quebec, Canada).

Transient transfection experiments were conducted in 6-well cluster plates as previously described (20, 23, 26) for adherent cell cultures. Transfections were conducted using the FuGENE 6™ (Roche Applied Science) or Lipofectamine 2000™ reagents (Invitrogen) method for 5–6 h according to the manufacturer's protocol with cells at 30–40% confluence. Cells were exposed to fresh complete culture medium for 4–16 h and, where indicated, synchronized for 1–3 h in 1% serum containing culture medium prior to experimentation. Transfection efficiencies were controlled in all experiments by cotransfection with 0.1 μg of pHSV-TK *Renilla* luciferase reporter vector (Promega, Madison, WI). The total amount of DNA/well was kept constant by the addition of carrier DNA. Luciferase values, expressed as enhanced relative light units, were measured in a Lumat LB 9507 dual-channel luminometer (EG&G, Stuttgart, Germany) and normalized to the levels of *Renilla* luciferase and cellular protein (bicinchoninic acid procedure; Pierce).

Extraction of Nuclear Proteins and EMSA Experiments—Confluent control and treated cells in 4-well cluster plates ($3–5 \times 10^6$ cells/well) were carefully scraped into 1.5 ml of ice-cold PBS and pelleted by brief centrifugation. Nuclear extracts were prepared as previously described (23, 26).

Double-stranded oligonucleotides containing wild-type and mutant sequences from the MMP-1 promoter (−2008 to −1972) were obtained from Invitrogen custom synthesis services, annealed in 100 nM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM EDTA buffer 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). The sense sequences of the oligos tested were as follows: NF-κB/c/EBP-like site MMP-1 promoter (−2008 to −1972): wt, 5′-TGA CGT CTT AGG CAA AAT CCT GTC CAA TCA CAG ATG-3′; 5′-TGA CGT CTT AGG CAA TTT CCT GTC CGG TCA CAG ATG-3′; 5′-TGA CGT CTT AGG CAA AAT CCT GTC CGG TCA CAG ATG-3′. In addition, consensus oligos were used routinely to assess cellular levels of NF-κB and c/EBP family members under basal and experimental conditions: 5′-AGC TTG GGG TAT TTC C

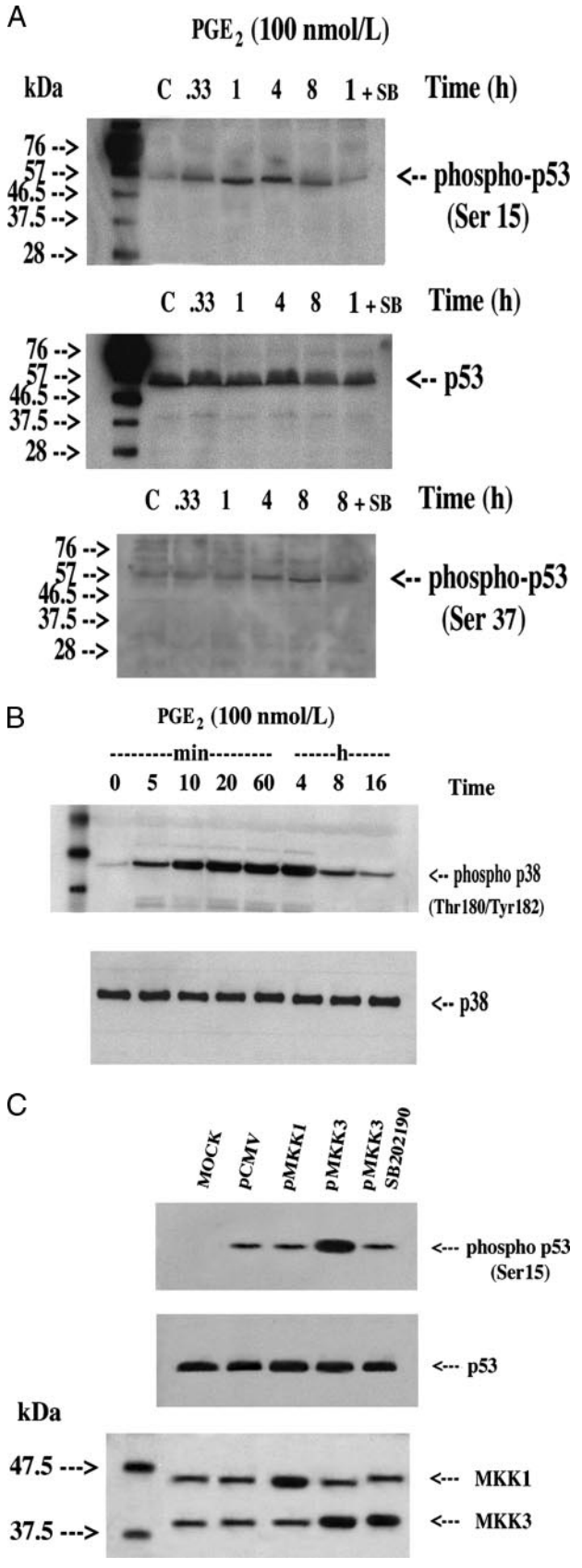

FIGURE 1. **PGE$_2$-dependent phosphorylation of p53 at Ser^{15} is a p38 MAP kinase-mediated process.** HSF, quiescent and synchronized in DMEM plus 1% FCS, were treated with 100 nmol/liter PGE$_2$ for varying time periods (0 up to 8 h as indicated) and then extracted for protein. In some experiments, PGE$_2$ was coincubated with 100 nmol/liter of SB202190 (*SB*), a preferential p38 MAP kinase inhibitor. 50 μg of protein were analyzed for [phospho-Ser^{15}]p53, total p53, and [phospho-Ser^{37}]p53 as in *A*, or *B* for total and phospho-p38 MAP kinase by Western blotting using specific affinity-purified polyclonal antibodies as described under "Experimental Procedures." In *C*, SN7-HSF were transfected with 1 μg each of pCMV vector, pCMV-pMMK1, or pCMV-MMK3 as described under "Experimental Procedures," and the cells were allowed to recover in complete medium for another 16 h. After quiescence and synchronization in DMEM plus 1% FCS for 3 h, cells were extracted for protein, and 50 μg were analyzed for [phospho-Ser^{15}]p53, p53, MKK1, and MKK3/6. In some cases SN7-HSF were transfected with pCMV-MMK3 in the presence of 100 nmol/liter of SB202190 and processed as described above. Blots are representative of 3–4 experiments.

(GCCGTATAACCmut) AG CCG-3′ and 5′-CTA GGG CTT GGC CAA T (TACCCmut)CT ATA TTC G-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 of ^{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 polyclonal anti-p52, C-Rel, p50, p65, and anti-c/EBPα, β, δ, and, γ (Santa Cruz Biotechnology, Inc.) were incubated for 10 min with the nuclear extracts prior to the addition of the radioactive probe. Binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis through 6% gels in a Tris borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

Statistical Analysis—All results were expressed as the mean ± S.D. of 2–5 experiments as indicated. Transfection experiments were performed in triplicate. Statistical treatment of the data were 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 that the null hypothesis was satisfied at <5%.

RESULTS

Prostaglandin E$_2$ Induces p53 Transactivation Through p38-mediated Phosphorylation of Serine 15—Preliminary studies showed that PGE$_2$ treatment of SN7-HSF transiently transfected with a p53RE (GADD45)-LUC reporter construct resulted in increased Luciferase activity that was judged to be p53-mediated because cotransfection of a dominant negative mutant of p53 abrogated this response (see below and Fig. 2*A*). Pursuing this line of investigation further, we observed that the eicosanoid induced a time-dependent increase in p53 Ser^{15} phosphorylation, with no change in overall p53 protein synthesis (Fig. 1*A*, *top* and *middle panels*). Endogenous levels of p53 were modest but stable under our culture conditions. The PGE$_2$-dependent Ser^{15} phosphorylation was apparently mediated by p38 MAP kinase as SB202190, a selective inhibitor of p38 MAP kinase activity, blocked the response (Fig. 1*A*, *top panel*). The time course of p38 MAP kinase Thr^{180}/Tyr^{182} phosphorylation in PGE$_2$-treated HSF was coincident with p53 Ser^{15} phosphorylation (Fig. 1*B*, *top* and *middle panels*). Furthermore, overexpression of a CMV-driven MKK3 construct, but not a MKK1 expression plasmid, mimicked the effects of PGE$_2$ in terms of Ser^{15} phosphorylation, an effect reversed by SB202190 (Fig. 1*B*, *lower panel*). Screening for other putative phosphorylation sites using a panel of anti-phospho-p53 antibodies revealed no other

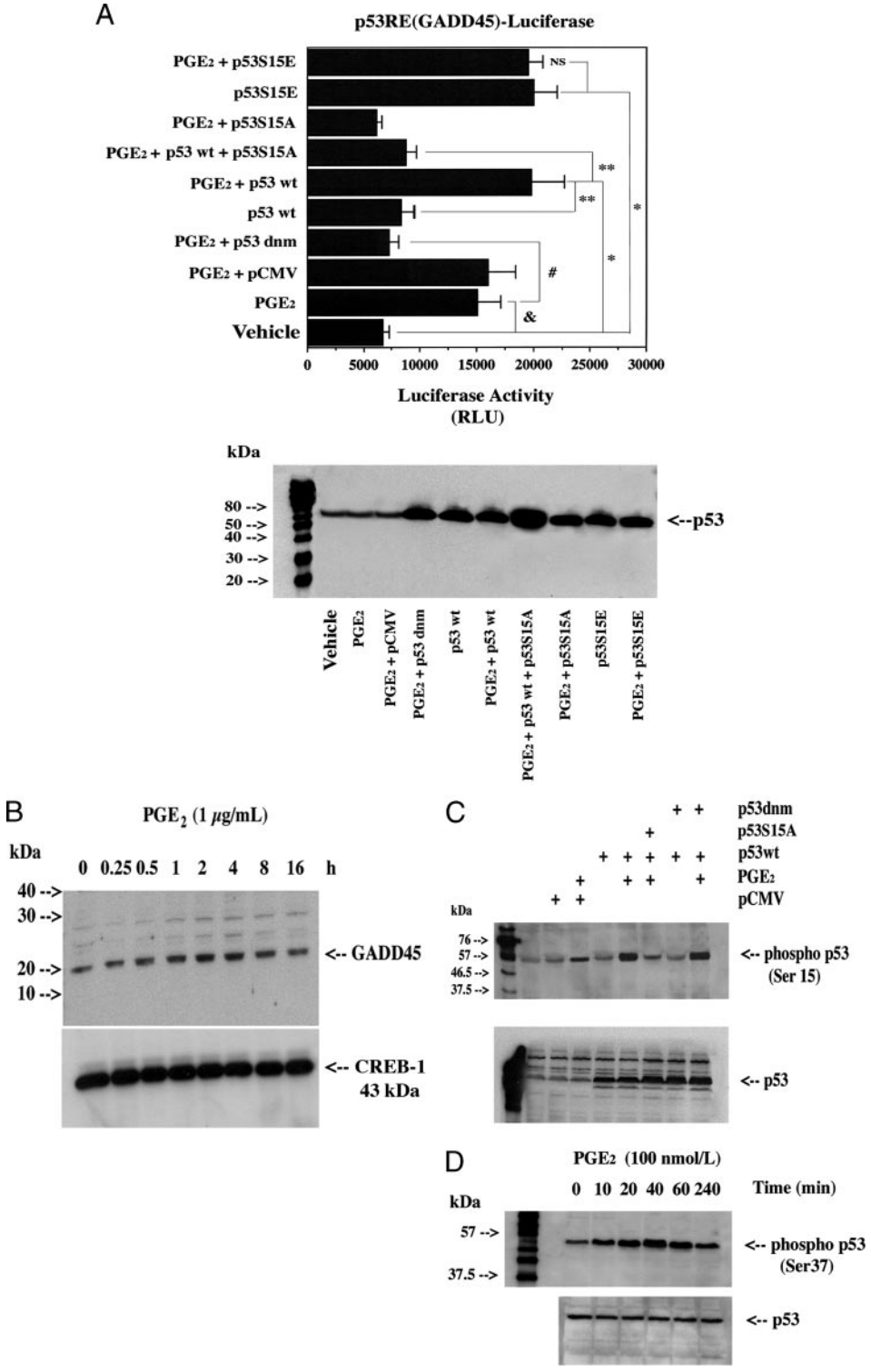

FIGURE 2. **PGE$_2$ stimulates p53 transactivational activity through specific Ser^{15} phosphorylation.** In *A*, SN7-HSF were transiently transfected with 1 μg/well of p53RE(GADD45)-luciferase reporter plasmid with or without the following cotransfected plasmids: pCMV (25 ng/well), p53 dnm (25 ng/well), p53wt (25 ng/well) with or without p53S15A (75 ng/well), p53S15A (25 ng/well), or p53S15E (25 ng/well). Cells were rinsed, and fresh complete medium was added for 4 h after which time the cells were rendered quiescent for 2 h in DMEM plus 1% FCS. PGE$_2$ (100 nmol/liter) was then added for 6 h as indicated. Cells were extracted for luciferase activity measurements (*top panel*) as described under "Experimental Procedures" and for total protein to perform Western analysis using an anti-p53 antibody (*bottom panel*). In *B*, quiescent/synchronized HSF (30×10^6 cells) were incubated with PGE$_2$ (1 μg/ml) for varying times as indicated and nuclei were isolated and extracted for protein. 150 μg were subjected to Western analysis and probed with anti-human GADD45 and anti-human CREB-1 (loading control) polyclonal antibodies. In *C*, SN7-HSF were transfected with the following plasmid mixes: pCMV (25 ng/well) or p53wt (25 ng/well) $\pm$ p53 dnm (75 ng/well) or p53S15A (75 ng/well) as indicated. PGE$_2$ (100 nmol/liter) was added for 5 h post-transfection, and 50 μg of protein from quiescent/synchronized cells were analyzed for total and phospho-Ser^{15} by Western blotting. In *D*, p53S15A (25 ng/well) transfected SN7-HSF were treated with PGE$_2$ (100 nmol/liter) for varying time periods (0–240 min) after which time extracted protein was subjected to Western analysis using total and [phospho-Ser^{37}]p53-specific antibodies. In *A*, results are expressed as mean $\pm$ S.D. of 3–5 determinations; Student's *t* test, (*) $p < 0.001$; (**) $p < 0.0037$; (#) $p < 0.025$; (&) $p < 0.01$; *NS*, not significant. *Renilla* values were typically between 15,850 and 19,570 RLU.

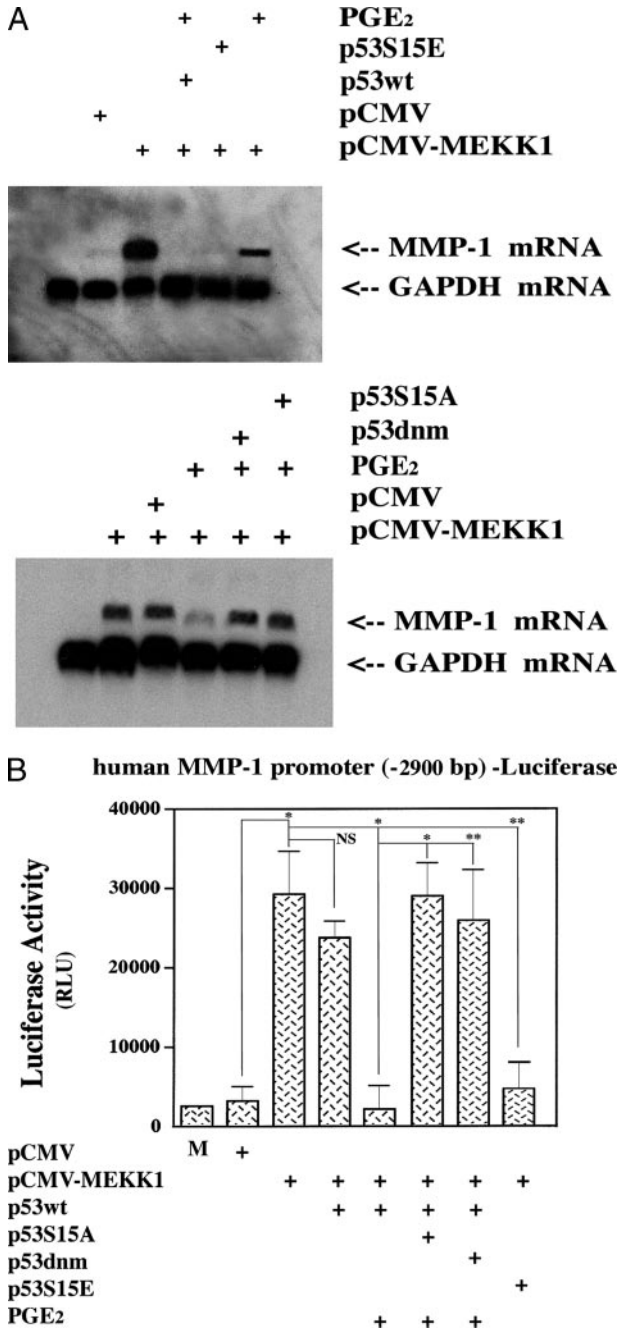

FIGURE 3. **p53 inhibition of MEKK1 induced MMP-1 mRNA expression, and MMP-1 promoter activity is mediated through PGE$_2$-induced specific p53 Ser^{15} phosphorylation.** In *A*, SN7-HSF were transiently transfected with 1 μg/well of pCMV-MEKK1 with or without the following cotransfected plasmids: *top panel*, pCMV (25 ng/well), p53wt (25 ng/well), or p53S15E (25 ng/well): *bottom panel*, pCMV (25 ng/well), p53 dnm (25 ng/well), or p53S15A (25 ng/well). PGE$_2$ (100 nmol/liter) was added during transfection as indicated and quiescent/synchronized cells were extracted for total RNA, and 5 μg were analyzed by Northern blot using specific probes for MMP-1 and GAPDH, as described under "Experimental Procedures." In *B*, SN7-HSF were transiently transfected with 1 μg/well of human MMP-1 promoter (2900 bp)-luciferase reporter plasmid with or without the following cotransfected plasmids: pCMV (100 ng/well), pCMV-MEKK1 (100 ng/ml), pCMV-MEKK1 + p53wt (25 ng/well), pCMV-MEKK1 + p53S15A (25 ng/well), pCMV-MEKK1 + p53wt (25 ng/well) + p53S15A (75 ng/well), pCMV-MEKK1 + p53wt (25 ng/well) + p53 dnm (75 ng/well), or pCMV-MEKK1 + p53S15E (25 ng/well). PGE$_2$ (100 nmol/liter) was added for 6 h post-transfection, and quiescent cells were assessed for luciferase activity as described under "Experimental Procedures." Results are expressed as mean ± S.D. of 2–5 determinations; Student's *t* test, (*) $p <$ 0.0005; (**) $p < 0.002$; *NS*, not significant. *Renilla* values were typically between 16,735 and 19,990 RLU.

PGE$_2$-dependent sites of phosphorylation with the exception of very weak and delayed modification of Ser^{37} (Fig. 1*A*, *lower panel*).

In an attempt to verify whether the Ser^{15} phosphorylation of p53 was sufficient to account for transactivation of a p53 response element(GADD45)-LUC reporter, we cotransfected HSF cell strain SN7 with wild-type and mutant constructs of p53 together with the reporter. Mutations, in the form of amino acid substitutions at Ser^{15}, were p53S15E and p53S15A, as well as the p53 dnm. As shown in Fig. 2*A*, substitution of glutamic acid (E) for serine (S) at position 15 resulted in a constitutively activated p53 in terms of transactivation of the target reporter; the addition of PGE$_2$ had no additive effect on the transactivational capacity of the constitutively active construct. In further experiments, p53wt expression alone was inactive in this regard being entirely dependent on PGE$_2$ treatment for the induction of reporter luciferase activity. The latter effect was abrogated by cotransfection with p53S15A and the dnm p53 at a ratio of 1:3 (wt/mutant) (Fig. 2*A*, *upper panel*). Comparable levels of wt and mutant p53 proteins were expressed in the absence or presence of PGE$_2$ (Fig. 2*A*, *bottom panel*). In principle, if PGE$_2$ activated a p53 response element of the GADD 45 promoter, then induction of the GADD45 protein, however transient, should follow. Indeed, we could demonstrate that an "inflammatory" concentration of PGE$_2$ caused a small but significant increase in GADD45 protein expression (Fig. 2*B*, *lower panel*) with no effect on the nuclear transcription factor CREB-1 (Fig. 2*B*, *lower panel*). Because it was incumbent upon us to show that the endogenous PGE$_2$-activated p38 MAP kinase activity had sufficient capacity to modify Ser^{15} of the transfected, overexpressed protein, we conducted careful examination of the phosphorylation state of transfected p53 (wt and mutants). Our results strongly suggested that Ser^{15} was indeed phosphorylated by PGE$_2$ treatments and expressing the p53S15A plasmid in our cell population diminished the phospho-Ser^{15}-dependent immunoreactivity (Fig. 2*C*). In this connection, p53S15A overexpression resulted in increased p38 MAP kinase-dependent Ser^{37} phosphorylation under our experimental conditions although the modification was not productive transcriptionally (Fig. 2*D*, see also Fig. 2*A*, *top panel*, *columns 7* and *8* from vehicle).

Prostaglandin E$_2$ Inhibits MEKK1 (Stress Kinase)-induced Interstitial Collagenase (MMP-1) Expression Through a p53-dependent Process; Role of p53 Ser^{15} Phosphorylation in Transcriptional Suppression of MMP-1 Promoter—To verify that PGE$_2$ can regulate a pathophysiologically relevant target gene and one ostensibly regulated by p53, we chose to study interstitial collagenase (MMP-1) (24). The latter metalloprotease is believed to play a critical role in the pathophysiology of inflammatory arthritides like RA and tumor metastasis with tissue invasion (15). Recent studies have suggested that the loss of regulatory control of MMP-1 in RA-affected synovial fibroblasts may be the result of inactivating mutations of p53 (18, 24, 28). In the latter cell type, a number of investigators have shown that proinflammatory cytokines stimulate MMP-1 expression in large part through activation of the MEKK1/MKK4/7/JNK signaling cascade (16, 29, 30). As shown in Fig. 3*A* (*top panel*), PGE$_2$-dependent inhibition of MEKK1-induced MMP-1 was

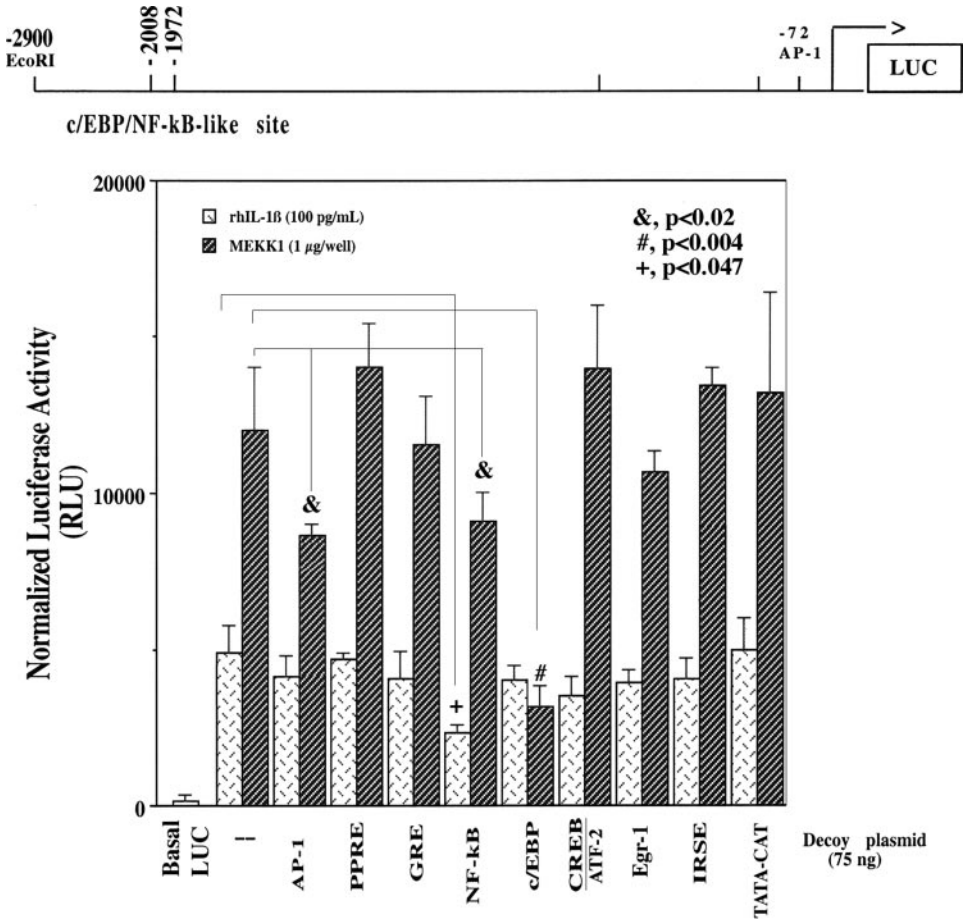

FIGURE 4. **MEKK1-induced MMP-1 promoter activity is largely dependent on c/EBPβ transactivation.** SN7-HSF were transiently transfected with 1 μg/well of human MMP-1 promoter (2900 bp)-luciferase reporter plasmid with or without 100 ng/well of MEKK1 and decoy plasmids containing the following enhancer elements (75 ng/well): AP-1, PPRE, GRE, NF-κB, c/EBP, CREB/ATF-2, Egr-1, ISRE, and empty vector (*i.e.* TATA-CAT). After 6 h, cells were exposed to fresh medium for 16 h after which time cells were synchronized for 3 h in 1% serum, and luciferase activity was measured as described under "Experimental Procedures." Basal luciferase (*Basal LUC*) refers to the RLU obtained with transfection of the empty pCMV (100 ng/well) construct, minus mock transfection values. Alternatively, synchronized cells, having been transfected only with the MMP-1 promoter (2900 bp)-luciferase reporter and decoys, were treated with 100 pg/ml of rhIL-1β for 16 h, and luciferase activities measured. Results are expressed as mean $\pm$ S.D. of 3–5 determinations in duplicate; Student's *t* test, (&) $p < 0.02$; (#) $p < 0.004$, (+) $p < 0.047$.

mimicked by overexpression of p53S15E and p53wt in the presence of PGE_2, but reversed when transfections were conducted in the presence p53 dnm or p53S15A (Fig. 3*A*, *lower panel*). To ascertain whether the PGE_2/p53-dependent effect on steady state levels of MMP-1 mRNA was manifested at the transcriptional level, we conducted transfection experiments using various human MMP-1 promoter constructs. In preliminary studies, a region encompassing −2900 bp (EcoRI) 5′ to the transcription start site, was shown to harbor p53-responsive sequences in HSF. As shown in Fig. 3*B*, p53wt-dependent reduction of MEKK1-induced MMP-1 promoter-driven luciferase activity required the addition of PGE_2; additions of either p53dnm or p53S15A to the plasmid mix, abolished the increase in luciferase activity. Transfection with p53S15E alone was sufficient to abolish MEKK1-induced MMP-1 promoter luciferase activity. The latter results were indistinguishable from those obtained when −3300 (PstI) and −4372 (HindIII) MMP-1 reporter plasmids were employed under identical experimental conditions (data not shown).

Identification of a c/EBP-NF-κB-like Element (−2008 to −1972 bp) in the MMP-1 Promoter-mediating MEKK1 (Stress Kinase)-induced Transcriptional Activation—The human MMP-1 promoter does not harbor copies of the 10-bp diad p53 DNA binding motif (31), suggesting that PGE_2-dependent p53 suppression of MEKK1-induced transcription occurs through some as yet undefined indirect mechanism in our cell cultures. As a first approach, we attempted to identify regions of the MMP-1 promoter responsive to MEKK1 activation by deletion analysis and found that responsive sequences were primarily distal; fold induction pCMV-MEKK1-induced MMP-1-promoter luciferase activity; −103 bp construct, 0.92 $\pm$ 0.23-fold; −512 bp, 0.86 $\pm$ 0.39-fold; −1600 bp, 1.19 $\pm$ 0.45-fold; −2900 bp, 4.6 $\pm$ 0.54-fold*; −3500 bp, 3.9 $\pm$ 0.83-fold*; −4372 bp, 3.05 $\pm$ 0.69-fold*, n = 3–5 determinations, *, $p < 0.001$ pCMV-MEKK1 *versus* pCMV). Cotransfection experiments with the −2900 (EcoRI) MMP-1 promoter construct (giving the most pronounced inducibility), pCMV-MEKK1, and different enhancer element decoy plasmids (see Fig. 4), revealed that the c/EBP decoy transfection decreased MEKK1 increases in MMP-1 promoter activity by 77.6% ($p < 0.004$, $n = 3$–5) whereas the AP-1 and NF-κB decoys appeared to have more modest effects in this regard (AP-1, 27.4%; NF-κBp65, 25.6% $p < 0.02$). For purposes of comparison, we conducted similar experiments with rhIL-1β (instead of MEKK1 transfection) and found that the proinflammatory cytokine induced promoter activity a maximum of 1.83 $\pm$ 0.33-fold; only the NF-κB plasmid reduced the rhIL-1β induction significantly (Fig. 4).

The strong inhibitory effects of the c/EBP decoy under MEKK1 activation, suggested that enhancer elements and *trans*-acting factors with known binding affinities for c/EBP sequences, may be involved in the induction of the MMP-1 promoter. In this regard, the human MMP-1 promoter harbors a c/EBP-NF-κB-like site at −2008 to −1972 bp (31), and point mutation analysis was conducted to assess the contribution of these elements in promoter activation. As shown in Table 1, mutating the CCAAT box element, resulted in a 57% drop in luciferase activity *versus* the wt promoter construct whereas mutating the NF-κB-like sequence abrogated 34% of total wt reporter activity. Double mutations reduced activity by almost

TABLE 1

NF-κB-c/EBPβ-like site in human MMP-1 promoter mediates MEKK1 (stress kinase)-induced transcriptional activation

SN7 human synovial fibroblasts at 30–50% confluence were transiently transfected with FuGENE 6 for 6 h with 1 μg/well of human MMP-1 promoter (−2900 bp, EcoRI)-luciferase construct (pGL3) either in its wt or mutant form (see above) together with 100 ng of pCMV-MEKK1 or 100 ng of pCMV (control) and 50–100 ng of pHSV-TK *Renilla* luciferase (transfection efficiency). pCMV-MEKK1-induced firefly luciferase activities were monitored 16 h post-transfection and subtracted from values obtained with the empty expression vector (i.e., pCMV) and then expressed as a ratio with *Renilla* luciferase values as denominator (i.e., fold-induction). Fold-induction values were expressed as mean ± S.D. from 3–5 determinations. Typical values for pCMV-MEKK1 promoter induction ranged from 3.68×10^4 to 1.31×10^5 RLU while background induction with pCMV ranged from 6244 to 8801 RLU. *Renilla* values were typically between 15,560 and 23,590 RLU.

−2008 NF-κB c/EBP −1972
5′-TGA CGT CTT AGG CAA TTT CCT GTC CAATCA CAG ATG-3′; wild-type

Promoter form	MEKK1 activation fold-induction
Wild-type	5.15 ± 0.66
CCAAT → CCGGT	2.23 ± 0.47[a]
ATTTC → AAATC	3.38 ± 0.54[b]
Double mutant	1.49 ± 0.42[a]

[a] $p < 0.004$.
[b] $p < 0.0295$ *vs* wild-type promoter construct.

TABLE 2

pSCT-LAP (c/EBPβ) induction of the human MMP-1 promoter activity: Role of NF-kB-c/EBPβ-like site

SN7 human synovial fibroblasts at 30–50% confluence were transiently transfected with FuGENE 6 for 6 h with 1 μg/well of human MMP-1 promoter (−2900 bp, EcoRI)-luciferase construct (pGL3) either in its wild-type (wt) or mutant form (see above) together with 100 ng of pSCT-LAP or 100 ng of pSCT (control) and 50–100 ng of pHSV-TK *Renilla* luciferase (transfection efficiency). pSCT-LAP-induced firefly luciferase activities were monitored 16 h post-transfection and subtracted from values obtained with the empty expression vector (i.e., pSCT) and then expressed as a ratio with *Renilla* luciferase values (i.e., fold-induction). Fold-induction values are expressed as mean ± S.D. from three determinations. Typical values for pSCT-LAP promoter induction ranged from 2.76×10^4 to 5.62×10^4 RLU while background induction with pSCT ranged from 7589 to 8157 RLU. *Renilla* values were typically between 14,950 and 21,226.

−2008 NF-κB c/EBP −1972
5′-TGA CGT CTT AGG CAA TTT CCT GTC CAATCA CAG ATG-3′; wild-type

Promoter form	pSCT-LAP (c/EBPβ) activation fold-induction
Wild-type	2.34 ± 0.45
CCAAT → CCGGT	1.21 ± 0.36[a]
ATTTC → AAATC	2.18 ± 0.47
Double mutant	1.17 ± 0.19[a]

[a] $p < 0.033$ *vs* wild-type promoter construct.

3.5-fold to near pCMV levels. To further assess the important role of c/EBP in promoter induction, we performed cotransfection experiments with 100 ng of pSCT-LAP (c/EBPβ expression vector) and 1 μg of −2900 MMP-1 promoter-luciferase construct. As shown in Table 2, ectopically expressed c/EBPβ caused a 2.34 ± 0.45 increase in promoter activity *versus* pSCT vector while mutating either the CCAAT box, the NF-κB site or both resulted in 1.21 ± 0.36-, 2.18 ± 0.47-, or 1.17 ± 0.19-fold increases, respectively.

MEKK1 (Stress Kinase)-induced Binding of c/EBPβ:c/EBPβ Homodimers and c/EBPβ:p65/RelA Heterodimers to the c/EBP-NF-κB-like Element (−2008 to −1972 bp) in the MMP-1 Promoter—To assess the nature and composition of putative transacting factors binding to the target sequences, which were presumably subject to PGE$_2$/p53 regulation, we performed gel-shift/super gel-shift analyses. We observed that MEKK1 overexpression induced strong specific binding to the cognate ^{32}P-labeled c/EBP-NF-κB-like oligo after 12–14 h post-transfection (Fig. 5*A*), a time course that proved to be optimal (data not shown). Supershift analysis using c/EBP isoform antibodies revealed that MEKK1 stimulated primarily the c/EBPβ isoform of the transcription factor, and, interestingly, essentially all of the ^{32}P-oligo was supershifted to a band near the top of the gel. However, the presence of a consensus-like NF-κB p65 site just 5′ to the CCAAT sequence suggested that heterodimers containing c/EBPβ and NF-κB could produce the same supershift patterns observed in Fig. 5*A*; interactions between NF-κB and c/EBP have been noted in previous studies (32). As such, preliminary gel-shift/super gel-shift studies were conducted to address whether or not overexpression of MEKK1 could induce NF-κB in our cell cultures. Indeed we observed a preponderant isoform of NF-κB p65 (Fig. 5*B*, *lower left*) and a slight but discernible shift with anti-p50. The addition of PGE$_2$ did not inhibit the MEKK1-induced stimulation of NF-κB p65 oligonucleotide binding (Fig. 5*B*, *lower right*).

In an attempt to resolve this conundrum, we generated wt, c/EBP, NF-κB, and double mutant ^{32}P-oligos of the c/EBP-NF-κB-like site at −2008 to −1972 bp and conducted gel-shift analysis with MEKK1-treated cell nuclear extracts. Reducing autoradiographic exposure times allowed the identification of 7 bands; 3 of which were apparently nonspecific (NS) and 4 specific binding species assigned arbitrarily as BP1-BP4 (data not shown). The use of mutant oligos permitted triage of the bands as shown in Fig. 5*C*. With the c/EBPmut (lane 5), a single, specific fast moving binding species was observed (identified as BP1). Using a ^{32}P-oligo with a mutated NF-κB site (lane 6), a more complex banding pattern was observed, identified as BP1, BP2, BP3, and BP4, where BP1 was noticeably reduced. Double mutant oligos were essentially unbound (lane 7). In supershift studies using the wt oligo, an anti-c/EBPβ antibody displaced the binding to near completion (as per Fig. 5*A*), whereas an anti-p65 antibody shifted B1 (Fig. 5*C*).

Prostaglandin E$_2$ Inhibits MEKK1 (Stress Kinase)-induced Protein Binding to the c/EBP-NF-κB-like Element Through a p53-dependent Process: Role of p53 Ser^{15} Phosphorylation—The addition of PGE$_2$ caused near complete inhibition of MEKK1-induced binding to the ^{32}P-labeled c/EBP-NF-κB-like oligonucleotide, as did cotransfection of the p53wt construct in the presence of the prostaglandin (Fig. 6*A*). The latter inhibitory patterns were reversed by the addition to the plasmid mixture of 3-fold excess of p53dnm and p53S15A.

The gel-shift studies suggested that PGE$_2$/p53 either suppress MEKK1-induced c/EBPβ biosynthesis or block the transactivational capacity of c/EBPβ. In this connection, previous studies indicate that MAP kinase-dependent phosphorylation at threonine (Thr) 235 is necessary for full transactivational activity of c/EBPβ at sites of target promoters (33). Alternatively, p53 (*e.g.* PGE$_2$-activated) could interact with c/EBPβ or induce proteins that associate with c/EBPβ to regulate transcriptional activation of target genes (34). As shown in Fig. 6*B*, the cellular level of c/EBPβ is unaffected by MEKK1 over expression with or without PGE$_2$/p53 treatments (lower panel). Furthermore, MEKK1 induced Thr^{235} phosphorylation of c/EBPβ is not significantly inhibited by PGE$_2$/p53 (upper panel). Therefore we tested the possibility that p53 regulates

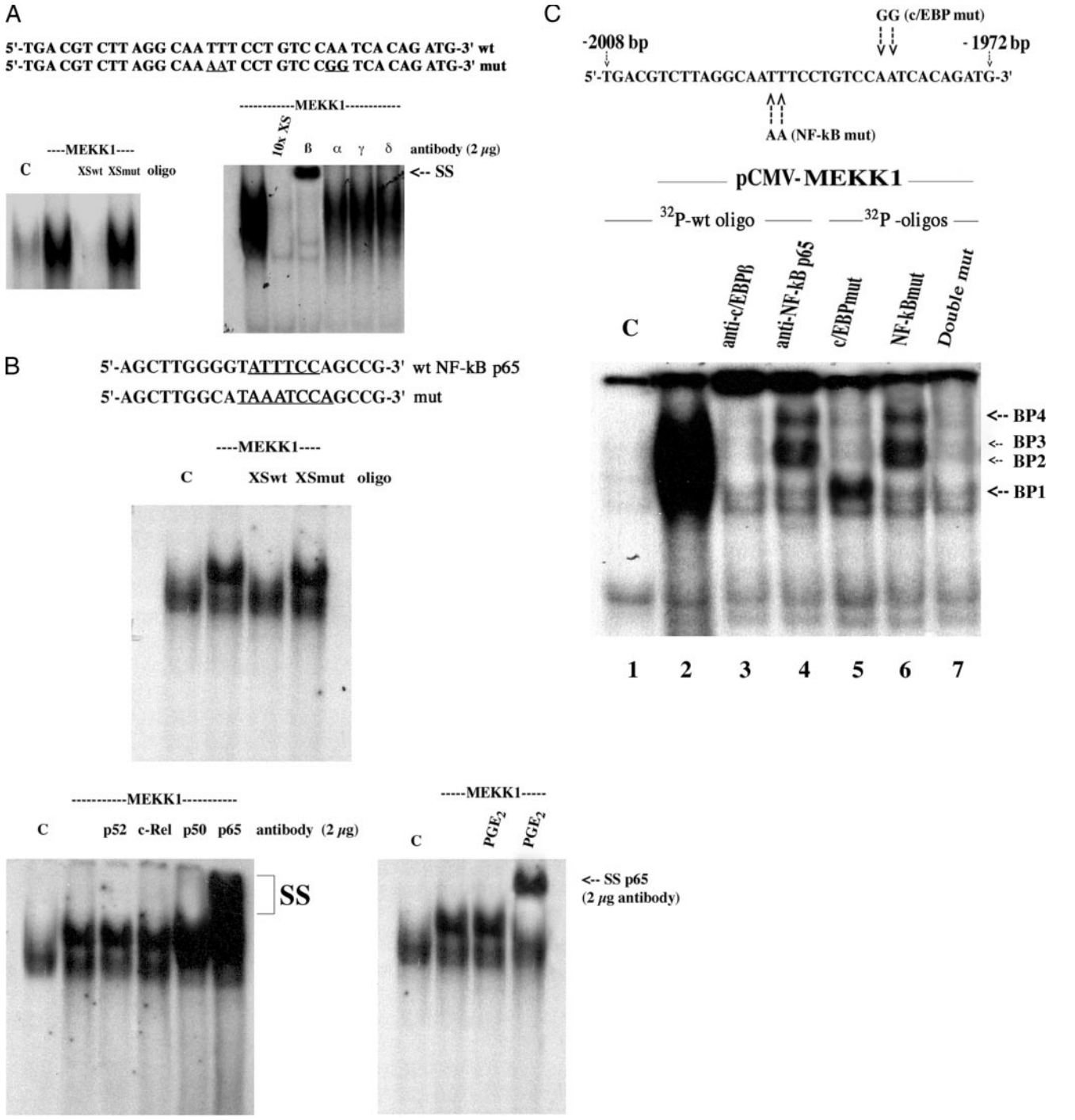

FIGURE 5. **MEKK1 (stress kinase)-induced binding of c/EBPβ:c/EBPβ homodimers and c/EBPβ:p65/RelA heterodimers to the c/EBP-NF-κB-like element (−2008 to −1972 bp) in the MMP-1 promoter.** In *A*, SN7-HSF were transiently transfected as described above with 100 ng/well of pCMV (control, *C*) or pCMV-MEKK1 and nuclear extracts from quiescent cells were subjected to gel-shift procedures as described under "Experimental Procedures" using a wt and mutant (mut) ^{32}P-labeled c/EBP-NF-κB-like probe encompassing a −2008 to −1972 sequence of the MMP-1 promoter as indicated (*left panel*). Super gel-shift analysis was conducted using the wt ^{32}P-labeled probe and specific antibodies to α, β, γ, or δ c/EBP (*right panel*). In *B*, 100 ng/well of pCMV (control, *C*), pCMV-MEKK1 or pCMV-MEKK1 + PGE_2 (100 nmol/liter)-treated nuclear extracts were subjected to gel-shift using a consensus wt and mut ^{32}P-labeled NF-κB p65 probe as indicated. Super gel-shift analysis was conducted using the wt ^{32}P-labeled probe and specific antibodies to p52, c-Rel, p50, and p65/RelA. In *C*, 100 ng/well of pCMV (control, *C*) or pCMV-MEKK1-treated nuclear extracts were subjected to gel-shift using the ^{32}P-labeled c/EBP-NF-κB-like probe or probes mutated at the NF-κB p65 or c/EBPβ or both sites as indicated. Super gel-shift analysis was conducted using the wt ^{32}P-labeled probe and specific antibodies to c/EBPβ and NF-κBp65. *BP*, binding protein. Data are representative of 3–4 experiments.

c/EBPβ-dependent promoter transactivation directly or indirectly (*i.e.* through a c/EBPβ-interacting protein controlled by p53/PGE_2). We chose a strategy used previously (32, 34), in which a construct containing a single copy of a c/EBP site (from c-Fos SRE) fused to a TATA-luciferase reporter, was cotransfected with the C/EBPβ expression vector with or without p53. As shown in Table 3, pSCT-LAP (c/EBPβ) induced a 4.93 ± 0.47-fold (over pSCT) increase in reporter activity 6–8 h after

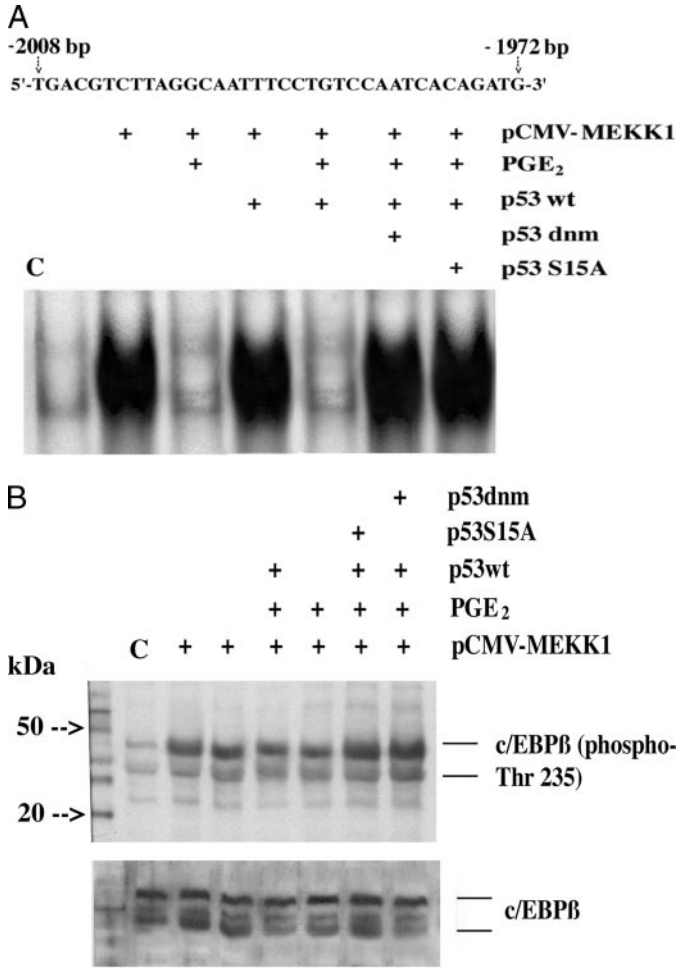

FIGURE 6. **Prostaglandin E_2 inhibits MEKK1 (stress kinase)-induced protein binding to the c/EBP-NF-κB-like element through a p53-dependent process: Role of p53 Ser^{15} phosphorylation.** SN7-HSF were transfected with 100 ng/well of pCMV (control, *C*) or pCMV-MEKK1 and p53wt, p53dnm, or p53 S15A (25 ng/well each) in the presence or absence of with PGE_2 (100 nmol/liter) as illustrated. Quiescent cells were extracted for nuclear protein and gel-shift analysis was conducted with the ^{32}P-labeled c/EBP-NF-κB-like probe encompassing a −2008 to −1972 sequence of the MMP-1 promoter (*A*). In *B*, following transfection procedures, quiescent cells were extracted for total cellular protein and Western analysis was conducted using anti-phospho-(Thr^{235}) c/EBPβ (*upper panel*) or anti-c/EBPβ (*lower panel*). Data are representative of three experiments for *A* and two analyses for *B*.

transfection, whereas p53wt + PGE_2, p53S15A, p53S15E, and pCREB-1 alone were without significant effect. However, when pSCT-LAP was cotransfected with p53wt + PGE_2 or p53S15E, reporter activity was reduced by 88%: p53S15A alone or with PGE_2 and pCREB-1 had no significant effect on c/EBPβ-induced reporter activity.

DISCUSSION

There is by now wide agreement that p53 plays an important function in maintaining cellular/tissular homeostasis by sensing DNA structural anomalies and orchestrating the appropriate cellular responses, processes dependent on p53 stability, and transcriptional activity (reviewed in Refs. 1 and 2). A convincing body of evidence maintains that Ser phosphorylation in the N-terminal transactivation domain plays a critical role in this regard and is essential in promoting interactions with important transcriptional coactivators (*e.g.* p300/CBP) and TATA box-binding proteins (35, 36). In response to UV (base-damaging) and IR (DNA strand breaks) radiation in immortal cell lines like MCF-7 cells, p38 MAP kinase-dependent phosphorylation occurs at Ser^{33} and Ser^{46}, an obligatory step in the coordination of phosphorylation of Ser^{15} and Ser^{37} and subsequent apoptosis (12). The latter report appears consistent with the notion that multiple integrated N-terminal phosphorylation of p53 by p38 MAP kinase is necessary for activating an irreversible cascade leading to apoptosis. However, genotoxic stress caused by nitric oxide (chondrocytes) or chemotherapeutic agents (NIH-3T3, Saos-2 cultures) results in p38 MAP kinase-dependent Ser^{15} phosphorylation, which apparently was entirely adequate to drive apoptosis (37). In a relevant physiological context, using cells derived from human explanted tissue and autocoids normally released during the inflammatory stress response, our results clearly indicated that increased p53 transactivational activity, both in a sequence-specific reporter and a target gene promoter, was sustained by specific Ser^{15} phosphorylation. Furthermore, overexpression of p53wt was insufficient to transactivate a target reporter in our cell cultures and required PGE_2-dependent activation of p38 MAP kinase for efficient Ser^{15} phosphorylation and promoter transactivation. In support, a constitutively active p53 construct with a glutamic residue at amino acid 15, fully recapitulated the effects of Ser^{15} phosphorylation. The significance of polyphosphorylation of the N-terminal domain of p53 subsequent to certain forms of genotoxic stress remains to be determined but it is known that

TABLE 3

Suppression of c/EBPβ-specific transactivation by PGE_2/p53

SN7 human synovial fibroblasts at 30–50% confluence were transiently transfected using FuGENE 6 for 6 h with 1 μg/well of SRE (c-Fos) (5′-AGCTTGATTAGGACATCG-3′)-TATA-luciferase together with 100 ng of pSCT-LAP or 100 ng of pSCT (control) and 50–100 ng of pHSV-TK *Renilla* luciferase (transfection efficiency). pSCT-LAP-induced firefly luciferase activities were monitored 6–8 h post-transfection and subtracted from values obtained with the empty expression vector (i.e., pSCT) and then expressed as a ratio with *Renilla* luciferase values (i.e., fold-induction). When added alone, p53wt, p53S15A, p53S15E, and pCMV-CREB-1 (pCREB-1) were transfected at 100 ng/well; when co-transfected with pSCT-LAP (c/EBPβ), the plasmids were added at 300 ng/well. PGE_2 (100 ng/ml) was added during transfection and 6–8 post-transfection. Fold-induction values are expressed as mean ± S.D. from 3 determinations. Typical values for pSCT-LAP promoter induction ranged from 2.009×10^4 to 2.13×10^5 RLU while background induction with pSCT ranged from 6598 to 9153 RLU. *Renilla* values were typically between 13,988 and 18,125.

SRE (c-Fos) [5′-AGCTTGATTAGGACATCG-3′]-TATA-Luciferase	
Plasmid/treatment	**Fold-induction**
pSCT-LAP (c/EBPβ)	5.93 ± 0.47
Serum 20%	9.66 ± 2.06
PGE_2 (1 μg/ml)	1.01 ± 0.3
p53wt + PGE_2	0.89 ± 0.27
p53S15A + PGE_2	1.07 ± 0.41
p53S15E	0.96 ± 0.32
pCREB-1	1.12 ± 0.35
pSCT-LAP (c/EBPβ) +	
PGE_2 (1 μg/ml)	1.91 ± 0.37[a]
p53wt + PGE_2	0.99 ± 0.41[b]
p53S15A	5.11 ± 0.63[ns]
p53S15A + PGE_2	4.89 ± 0.57[ns]
p53S15E	1.66 ± 0.22[a]
pCREB-1	6.12 ± 0.75[ns]

[a] $p < 0.0014$.
[b] $p < 0.0008$.
ns, not significant.

Ser^{15} phosphorylation is adequate to inhibit the association of the MDM2 with p53 and increase the stability/activity of the protein (10). In a somewhat oblique reference, a recent report found that a single N-terminal-truncated p53 subunit was enough to abolish the transcriptional activity of the p53 tetramer. However, three p53 subunits with mutations in the SV40T and DNA binding domains were required to inhibit p53 transactivation (39). Of possible significance, Ser^{37} was not a primary target for PGE_2-activated p38 MAP kinase except when the cells were transfected with the mutant p53 S15A, in which case the presence of an alanine at amino acid 15 precluded phosphorylation and thus the secondary Ser^{37} was modified. An examination of the flanking amino acid sequences reveals that the Pro-Leu-Ser^{15}-Gln-Glu-Thr is a preferred site for phosphokinase activity compared with the Pro-Leu-Pro-Ser^{37}-Gln-Ala-Met (38).

Increased MMP expression and activity is a hallmark of tumor invasion, metastasis, matrix destruction in arthritis and synovial cell hyperproliferation (15). Though much has been reported about MMP up-regulation, work from our laboratory has provided evidence that PGE_2 is a major negative physiological regulator of MMP expression in synovial tissue (22, 39, 40). Given the pleiotropic nature, finely controlled and temporal precision of PGE_2 action, it is likely that the eicosanoid inhibits MMP-1 expression or other target genes for that matter by more than one signaling pathway. Nevertheless, this is the first report that PGE_2 regulates p53 transactivational activity, which potently reduces stress kinase-induced *MMP-1* gene expression through promoter suppression. Because the MMP-1 promoter harbors no p53 cognate sites (31), it has been suggested that p53 may inhibit MMP-1 expression in serum-stimulated foreskin fibroblasts through interaction with AP-1 (29, 31), a putative transcriptional activator of the MMP-1 promoter in a number of cell lines (16, 29–31). Somewhat paradoxically however, PGE_2 is a (the) potent activator of AP-1 in HSF and can transactivate the proximal AP-1 loci of the 103-bp MMP-1 promoter construct (22). Indeed, PGE_2 is more active than phorbol esters in this regard and IL-1β-dependent induction of the MMP-1 promoter (AP-1 loci) is actually mediated by PGE_2. Our present study resolves this paradox to the extent that stress kinase-activated MMP-1 expression in HSF occurs largely through c/EBPβ/NF-κBp65 promoter transactivation. Our study is of course not the first report of functional and physical interactions between c/EBPβ and NF-κB but represents a novel observation with regards to stress-induced MMP-1 regulation. Furthermore, the necessary contribution of NF-κBp65 to an otherwise c/EBPβ-regulated promoter is in agreement with previous work indicating that, in promoters with a c/EBPβ enhancer format, NF-κBp65:c/EBPβ association is required for full promoter transactivation (32, 41). This is ostensibly the result of the obligate role of NF-κBp65 in co-activator protein interactions (*e.g.* p300/CBP, p/CAF) and transcriptional complex formation (41). It is however, not possible to rule out an accessory role for AP-1 in MEKK-1-induced MMP-1 expression, because in HSF the canonical AP-1 site (−72), though occupied in unstimulated cells, demonstrates increased binding of AP-1 members such as c-Jun/c-Jun and c-Jun/c-Fos dimers as opposed to JunD/JunD and JunD/c-Jun dimers in unstimulated cells (22). It is quite reasonable to speculate that c/EBPβ homodimers and c/EBPβ/NF-κBp65 heterodimers could physically interact with AP-1 and binding proteins of the transcriptional enhancer complex (see Ref. 42). This may account for the modest but significant reduction of MEKK-1-induced MMP-1 promoter activity in the presence of an AP-1 decoy.

It is noteworthy that in the absence of cognate enhancer elements, p53 can modulate transcription at the promoter level by protein-protein interactions with other transacting factors. For example, the activation domain of p53 interacts with the TATA box-binding polypeptide in Holo-TFIID and inhibits transcription (35). Furthermore, p53 negatively regulates induced cdc2 transcription by interaction with CCAAT site binding transcription factor NF-Y (43). In a more direct fashion, p53 was shown to regulate the Hsp70 promoter through protein-protein interaction with CCAAT-binding factor (CBF, *i.e.* c/EBP) at a c/EBP-like site in CHO cells (34). In the present study (Table 3 and Fig. 6*B*), we provided strong albeit circumstantial evidence that PGE_2-dependent p53 Ser^{15} phosphorylation was a *sine que non* for p53 suppression of MEKK1-induced MMP-1 promoter activation and that phosphopSer^{15}p53 probably interacts physically (direct) with c/EBPβ dimers (c/EBPβ/NF-κB heterodimers) or through a regulated p53 target protein. This seems like a reasonable conclusion because we found no evidence of [phospho-Ser^{15}]p53 acting on MEKK-1 stimulated cell signaling intermediaries, on the cellular level of c/EBPβ, or on the phosphorylation at Thr^{235} of c/EBPβ to its transcriptionally active form. As the data in Table 3 suggest, [phospho-Ser^{15}]p53 does not bind to c/EBPβ binding sites so that enhancer site blockade of c/EBPβ transactivation of the SRE-TATA-luciferase promoter is not likely. It is also improbable that [phospho-Ser^{15}]p53·c/EBPβ complexes simply function as repressors because, in principle, one should still detect DNA binding in our gel-shift experiments as in Fig. 6*A*. One important observation is that it seems only (tetrameric) transcriptionally competent p53 was active in terms of inhibiting DNA binding or suppressing transcription. This suggests that [phospho-Ser^{15}]p53 may be limiting c/EBPβ/c/EBPβ and c/EBPβ; NF-κBp65 access to cognate sites in the MMP-1 promoter and/or competing for TATA-binding proteins as has been shown previously (44). Lastly, a p53/PGE_2-regulated protein may associate with c/EBPβ and interestingly, the GADD family of proteins is known to bind c/EBPβ and suppress target promoter activation (45). We showed in the present study that PGE_2 stimulated GADD45 protein and promoter.

As demonstrated in this present study, PGE_2, at concentrations resulting in ~80% EP4 receptor occupancy (100 nmol/liter, Ref. 46), activated p53. At <100 nmol/liter, the eicosanoid does not induce appreciable apoptosis of HSF, as judged by a number of experimental parameters that define apoptotic events (*e.g.* annexin V binding by FACS analysis) (Ref. 47, data herein). However at 1 μmol/liter, still in the concentration range observed in severe inflammatory episodes, PGE_2 can induce ~70% of the HSF in culture to undergo apoptosis after 16–24 h of incubation (47). These observations may be related to PGE_2-induced $p16^{ink4a}$ expression (JunB-mediated) with the

accompanying suppression of the cyclin D1 promoter activity.[4] Thus, PGE_2 may serve as a molecular "bridge" between the inflammatory response and cellular transformation in hyperproliferative pathologies.

We believe these results to be of considerable clinical significance because of the widespread use of NSAIDS to treat OA and RA (possibly colon cancer) and the possibility that disease progression is exacerbated by such usage. The present observation that p53 transcriptional activity is induced by PGE_2 strongly suggests that NSAIDS may actually compromise p53 function. We believe that the cPLA2-COX-2-PGES axis is a central homeostatic control point used by certain cells to adapt to a stress-induced environment and thus should not be compromised.

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[4] J. A. Di Battista, unpublished observations.