Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor κB

Zhiyuan YU, Wenzheng ZHANG and Bruce C. KONE[1]

Departments of Internal Medicine and of Integrative Biology, Pharmacology and Physiology, The University of Texas Medical School at Houston, 6431 Fannin, MSB 4.148, Houston, TX 77030, U.S.A.

Prolific generation of NO by inducible nitric oxide synthase (iNOS) can cause unintended injury to host cells during glomerulonephritis and other inflammatory diseases. While much is known about the mechanisms of iNOS induction, few transcriptional repressors have been found. We explored the role of signal transducers and activators of transcription 3 (STAT3) proteins in interleukin (IL)-1β- and lipopolysaccharide (LPS)+interferon (IFN)-γ-mediated iNOS induction in murine mesangial cells. Both stimuli induced rapid phosphorylation of STAT3 and sequence-specific STAT3 DNA-binding activity. Supershift assays with a STAT3 element probe demonstrated that nuclear factor κB (NF-κB) p65 and p50 complexed with STAT3 in the DNA–protein complex. The direct interaction of STAT3 and NF-κB p65 was verified *in vivo* by co-immunoprecipitation and *in vitro* by pull-down assays with glutathione S-transferase-NF-κB p65 fusion protein and *in vitro*-translated STAT3α. Overexpression of STAT3 dramatically inhibited IL-1β- or LPS+IFN-γ-mediated induction of iNOS promoter-luciferase constructs that contained the wild-type iNOS promoter or ones harbouring mutated STAT-binding elements. In tests of indirect inhibitory effects of STAT3, overexpression of STAT3 dramatically inhibited the activity of an NF-κB-dependent promoter devoid of STAT-binding elements without affecting NF-κB DNA-binding activity. Thus STAT3, via direct interactions with NF-κB p65, serves as a dominant-negative inhibitor of NF-κB activity to suppress indirectly cytokine induction of the iNOS promoter in mesangial cells. These results provide a new model for the termination of NO production by activated iNOS following exposure to pro-inflammatory stimuli.

Key words: gene regulation, mesangial cell, promoter.

INTRODUCTION

NO is a potent effector molecule involved in numerous physiological processes, including neurotransmission, the control of vascular tone, inflammation and immunity. NO also serves important roles in glomerular function [1], and excessive NO production has been linked to several forms of glomerular injury [2,3]. Mesangial cells within the glomerulus contribute to the regulation of glomerular filtration, phagocytosis of immune complexes and the production of extracellular matrix. When activated by immunological or inflammatory stimuli, mesangial cells generate cytokines, chemokines and high-output NO.

NO production is governed by the activity of three nitric oxide synthase (NOS) isoforms. Both neuronal and endothelial NOS are generally expressed under basal conditions in selected cells and are typically calcium- and calmodulin-dependent. Inducible NOS (iNOS) is quiescent in most tissues until it is transcriptionally activated by immune stimuli to produce large amounts of NO [4]. The sustained flux of large amounts of NO produced by iNOS can result in cytotoxicity to both the host and the target cell. Accordingly, both positive and negative modulators have evolved to control tightly iNOS expression and to prevent untoward effects of excessive NO production. While much is known about the activation of iNOS transcription by cytokines and bacterial lipopolysaccharide (LPS), relatively little is known about how iNOS transcription might be constrained. Several stimuli, such as interleukin (IL)-1β [5], LPS and interferon (IFN)-γ [6], activate iNOS gene transcription in mesangial cells. Various signalling pathways and inducible transcription factors, including cAMP [5], c-Jun N-terminal kinase and p38 mitrogen-activated protein kinase [7], cAMP-response-element-binding protein (CREB) [5], CCAAT/enhancer-binding protein β [5] and nuclear factor κB (NF-κB) [8], have been implicated in iNOS gene activation in mesangial cells. In contrast, transforming growth factor-β [9], IL-13 [10] and Janus kinase (JAK) 2 [11] are known to inhibit iNOS activation in these cells.

Several inducible transcription factors exert complex control over the murine iNOS promoter. A pivotal role for the two κB sites, positioned at -85 to -76 and -971 to -962 in the murine iNOS promoter, has been confirmed using deletion analysis and selective base mutation of the binding sequences [12]. The murine iNOS promoter is also known to have at least one functional INF-γ-activated site (GAS) at -942 to -934, 5′-TTCCCCTAA-3′ (consensus sequence, TTCNNNTAA). Binding of signal transducers and activators of transcription (STAT) 1α to this site has been shown to transactivate the iNOS gene in LPS+IFN-γ-treated RAW 264.7 macrophages [13]. In other cell types, however, STAT1 suppresses iNOS gene expression [14].

Cytokine signal transduction is predominantly mediated through the JAK/STAT signalling pathway [15]. The JAKs, cytokine receptor-associated tyrosine kinases, phosphorylate the STAT family of latent transcription factors. Tyrosine phosphorylation by the JAKs or the Src family of tyrosine kinases promotes STAT homo- or hetero-dimerization and translocation of dimerized STAT to the nucleus. The dimerized STAT then alters transcription by binding to specific response elements

Abbreviations used: CBP, CREB-binding protein; CREB, cAMP-response-element-binding protein; EMSA, electrophoretic mobility-shift assay; GAS, INF-γ-activated site; GST, glutathione S-transferase; IFN, interferon; IL, interleukin; NOS, nitric oxide synthase; iNOS, inducible NOS; JAK, Janus kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; STAT, signal transducers and activators of transcription.

[1] To whom correspondence should be addressed (e-mail Bruce.C.Kone@uth.tmc.edu).

© 2002 Biochemical Society

in the promoters of target genes. When activated, STAT factors can stimulate or inhibit gene transcription, perhaps conditional on their interaction with heterologous transcription factors [16] and/or co-activators/co-repressors [17]. The STAT family participates in the regulation of genes involved in the acute phase response, inflammation, cell growth and differentiation. STAT3 is principally expressed in the kidney, liver and spleen. It is alternatively spliced to yield two isoforms, termed STAT3α and STAT3β. STAT3β lacks the 55 C-terminal amino acid residues of STAT3α and has seven additional amino acid residues at its C-terminus [18]. Studies in cell lines and genetically engineered animals have demonstrated important roles for STAT3 in promoting cell-cycle progression and cellular transformation and in limiting apoptosis [15].

Transcriptional regulation of eukaryotic genes often requires the co-operative or antagonistic action of several proteins. Protein–protein interactions have been demonstrated to be important for the ability of STAT proteins to regulate target gene transcription. Although activated by different pathways, activated STATs and NF-κB translocate into the nucleus and function either individually or co-operatively in regulating the expression of target genes [19–22]. In one example, STAT5β has been shown to inhibit NF-κB signalling by competing for limiting amounts of co-activators necessary for NF-κB-mediated gene transcription [22]. Conversely, STAT1 and STAT3 have been shown to serve as *trans*-activators capable of recruiting p300/CREB-binding protein (CBP) [23]. Relatively little is known, however, about how combinatorial interactions of transcription factors regulate iNOS gene transcription. In this report, we show that STAT3 can directly interact with NF-κB components and antagonize cytokine activation of the iNOS gene and a κB element reporter construct in mesangial cells. This interplay of transcription factors may represent an important cell-specific mechanism to down-regulate the inflammatory response and facilitate mesangial cell proliferation.

EXPERIMENTAL

Cell culture and reagents

Mouse mesangial cells (ATCC CRL-1927) were maintained in Ham's F12 plus Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5 % fetal bovine serum. RAW 264.7 macrophage cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % fetal bovine serum. Vehicle, IL-1β (10 ng/ml) or LPS (1 μg/ml) + IFN-γ (100 units/ml) was added to the cells as indicated in the text and Figure legends. Mouse recombinant IL-1β and IFN-γ were from R&D Systems (Minneapolis, MN, U.S.A.) and BioSource (Camarillo, CA, U.S.A.), respectively. Polyclonal antibodies recognizing STAT1, STAT3 (C20) and NF-κB p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). A polyclonal antibody specific for phospho-Tyr^{705}-STAT3 was from New England Biolabs (Beverly, MA, U.S.A.). A polyclonal antibody recognizing NF-κB p65 was from Upstate Biotechnology. Oligonucleotides were custom-synthesized by Genosys (The Woodlands, TX, U.S.A.). Lipofectamine 2000 reagent was from Invitrogen (Carlsbad, CA, U.S.A.). The Dual-Luciferase™ Reporter Assay System and the luciferase vectors pGL3-Basic and pRL-SV40 were from Promega. The BCA protein estimation kit was from Pierce Chemical. Glutathione–Sepharose 4B beads, pGEX-5X-3 and ECL® reagents were from Amersham Bioscience (Piscataway, NJ, U.S.A.).

Plasmids and site-directed mutagenesis

The STAT3α and STAT3β expression plasmids pSG5-STAT3α and pSG5-STAT3β were kindly provided by Dr David Tweardy (Baylor College of Medicine, Houston, TX, U.S.A.). The NF-κB reporter construct, p36B(−)(NF-κB)$_3$-luc, which contains three tandem copies of the κB-binding element (GGGGACTTTCCC) upstream of the simian virus 40 early promoter sequence and fused to the coding sequence for the luciferase gene [24], was provided by Dr Bharat Aggarwal (University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.A.). piNOS-luc, which contains the murine iNOS promoter/enhancer and a portion of exon 1 (nucleotides −1486 to +145) in pGL3-Basic, has been previously characterized [25]. To generate fusions with glutathione S-transferase (GST), a cDNA insert encoding murine NF-κB p65 was amplified by reverse transcriptase PCR from mesangial cell RNA and subcloned into pGEX-5X-3 at the *Eco*RI and *Not*I sites to maintain the appropriate reading frame and sequenced to verify its authenticity. Site-directed mutation of the −942 to −934 STAT-binding element (5′-TTCCCCTAA-3′, replaced with TGCCGACAA; mutations are underlined) and of the −879 to −871 STAT-binding element (5′-TTATTGGAA-3′, replaced with GGCTAAGGC) in piNOS-luc was accomplished by PCR splicing by overlap extension, using the wild-type iNOS promoter DNA as a template. The mutated iNOS promoters were cloned into pGL3-Basic to create the recombinant molecules piNOS-ΔSTAT3$^{-942/-934}$-luc and piNOS-ΔSTAT3$^{-879/-871}$-luc, which were sequenced to verify the presence of the desired mutations and the absence of spurious mutations.

Transient transfections

Mesangial cells or RAW 264.7 cells were seeded in 24-well plates and grown to 90–95 % confluency in complete medium without antibiotics and transfected the following day using the Lipo-Fectamine 2000 reagent following the manufacturer's protocol and a total of 1 μg/well of plasmid DNAs. The amount of transfected DNA was kept constant by addition of appropriate amounts of the parental empty expression vector. Transfection efficiencies were normalized by co-transfection with 20 ng/well of the *Renilla* luciferase expression plasmid pRL-SV40.

For *trans*-repression experiments 0.78 μg of piNOS-luc, piNOS-ΔSTAT3$^{-942/-934}$-luc, piNOS-ΔSTAT3$^{-879/-871}$-luc, p36B(−)(NF-κB)$_3$-luc or promoterless expression vector was co-transfected with 0.2 μg of pSG5-STAT3αpSG5-STAT3β, or insertless expression vector pSG5, along with 0.02 μg of pRL-SV40. After transfection (24 h), the medium was added with vehicle, IL-1β or LPS + IFN-γ. Another 24 h later, cell lysates for measurement of firefly and *Renilla* luciferase activities were prepared and firefly and *Renilla* luciferase activities in 100 μl lysate samples were measured as described previously in our laboratory [25]. In some experiments, nuclear extracts were prepared from the transfected cells for electrophoretic mobility-shift assay (EMSA). In pilot experiments, transfection efficiency was determined by transfection of pSV-β-galatosidase control vector (Promega, Madison, WI, U.S.A.) followed by staining with X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and cell counting. The transfection efficiency for both cell lines was reproducibly 40–50 %.

Preparation of whole-cell and nuclear extracts

For preparation of whole-cell lysates, cell monolayers were stimulated by IL-1β or LPS + IFN-γ for 15 min. The plates were then washed twice with ice-cold PBS, and the pellet was resuspended in lysis buffer (20 mM Hepes, pH 7.9, 20 mM NaF, 1 mM

© 2002 Biochemical Society

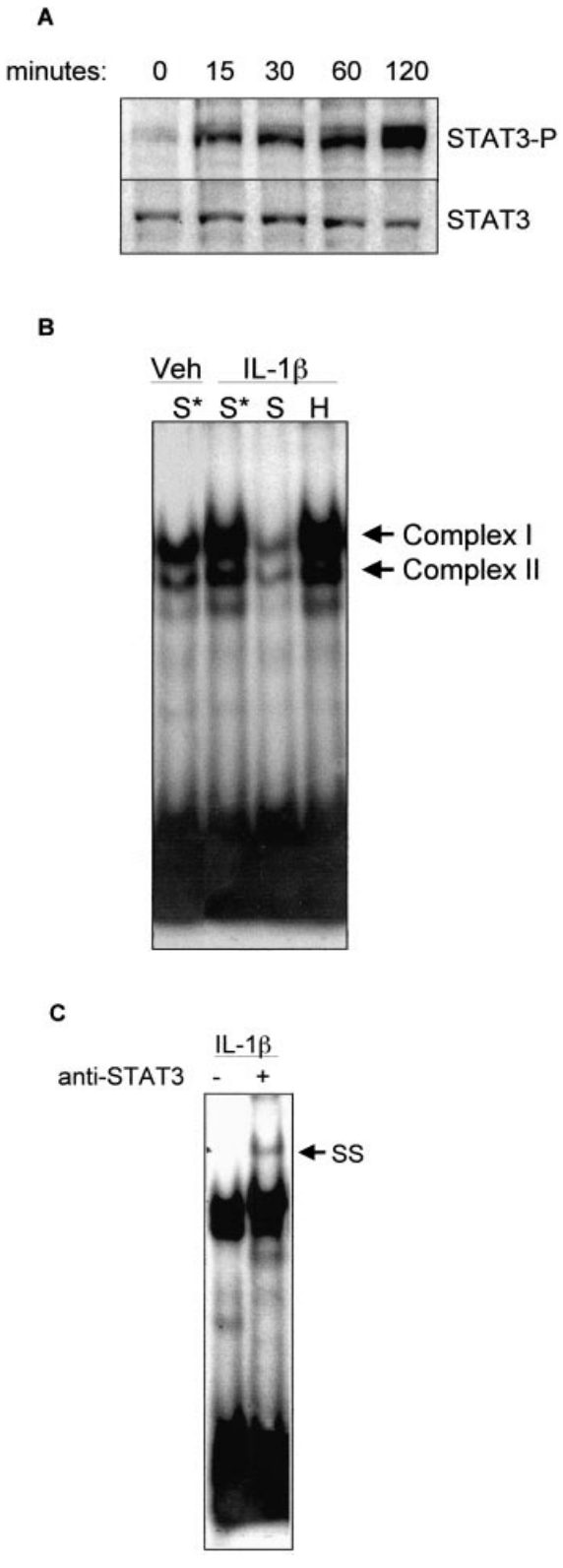

Figure 1 LPS and cytokines promote STAT3 phosphorylation and DNA-binding activity in nuclear protein extracts of mesangial cells

(**A**) Nuclear proteins extracted from mesangial cells that had been exposed to vehicle or LPS + IFN-γ for the indicated times were separated by SDS/PAGE, blotted and probed with a phospho-Tyr^{705}-STAT3-specific antibody or a polyclonal antibody directed against STAT3. Similar induction of tyrosine-phosphorylated STAT3 was evident after IL-1β treatment. (**B**) Nuclear extracts from vehicle-(Veh) or IL-1β-treated mesangial cells were subjected to EMSA with a ^{32}P-labelled oligomer encoding a STAT3 consensus binding site (S*). To demonstrate binding specificity, reactions were also conducted in the presence of a 50-fold molar excess of unlabelled STAT3 oligomer (S) or heterologous (H) oligomers. The autoradiogram is representative of four independent experiments performed on separate preparations of nuclear extracts. (**C**) Polyclonal antibody specific for STAT3 or a non-immune IgG was used in supershift (SS) experiments with nuclear extracts from IL-1β-treated (15 min) mesangial cells and the ^{32}P-labelled STAT3 oligomer. The autoradiograms are representative of three independent experiments performed on separate preparations of nuclear extracts.

Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 420 mM NaCl, 20% glycerol and protease inhibitor cocktail). After freezing in dry ice/ethanol bath and thawing on ice three times, the lysate was microcentrifuged at 15000 ***g*** for 20 min. The collected supernatant represented the whole cell extract. Nuclear extracts were prepared from time-paired control, IL-1β- or LPS + IFN-γ-treated mesangial cells as detailed in our earlier work [25,26].

EMSAs

The following double-stranded oligonucleotides from the murine iNOS promoter were used as probes and for competition studies (the sense strand is shown; consensus binding element underlined; u and d represent upstream and downstream): NF-κBu, 5′-$^{-978}$TGCTAGGGGGATTTTCCCTCTCTC^{-955}-3′; NF-κBd, 5′-$^{-92}$CCAACTGGGGACTCTCCCTTTGGG^{-69}-3′; GASu, 5′-$^{-948}$TTCCTTTTCCCCTAACACTG^{-929}-3′; GASd, 5′-$^{-837}$TTTGTTTCTCAGAACAGGGT^{-818}-3′. The probes were end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. A double-stranded nucleotide containing high-affinity double-stranded STAT3 consensus binding site 5′-GATCCTTCTGGGAATTCCTAGATC-3′ [27] was from Santa Cruz Biotechnology. Binding reactions were performed in 20 μl of solution for 30 min at room temperature by incubating 10 μg of nuclear extract protein with duplex DNA probe ($\approx 2 \times 10^5$ c.p.m.) in reaction buffer [13 mM Hepes, pH 7.9, 65 mM NaCl, 0.14 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 8% glycerol and 50 μg/ml poly(dI-dC)] in the presence or absence of a 50-fold molar excess of non-radiolabelled competitor oligonucleotides. For supershift assays, antibodies (2 μg) specific for STAT1, STAT3, NF-κB p50 or p65, or non-immune IgG were added to the binding reaction and incubated on ice for 10 min before the addition of labelled probe. Aliquots of the reactions were resolved on 5% native polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer. The gels were dried and exposed to X-ray film with an enhancing screen at −70 °C to detect the DNA–protein and DNA–protein–antibody complexes. Experiments were replicated a minimum of three times as indicated in the Figure legends.

Western blotting

Samples (20 μg) of nuclear, cytoplasmic or whole-cell extracts were resolved by SDS/PAGE, and the proteins were electrophoretically transferred to PVDF membranes (Hybond ECL; Amersham Bioscience). The blots were probed with an anti-STAT3 antibody (0.2 μg/ml) or an anti-phospho-STAT3 antibody (0.2 μg/ml) overnight at 4 °C. The blots were washed extensively with a solution containing 50 mM Tris, pH 8.0, 138 mM NaCl, 2.7 mM KCl and 0.05% Tween 20. The antigen–antibody complexes were detected by the ECL protocol using horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody.

© 2002 Biochemical Society

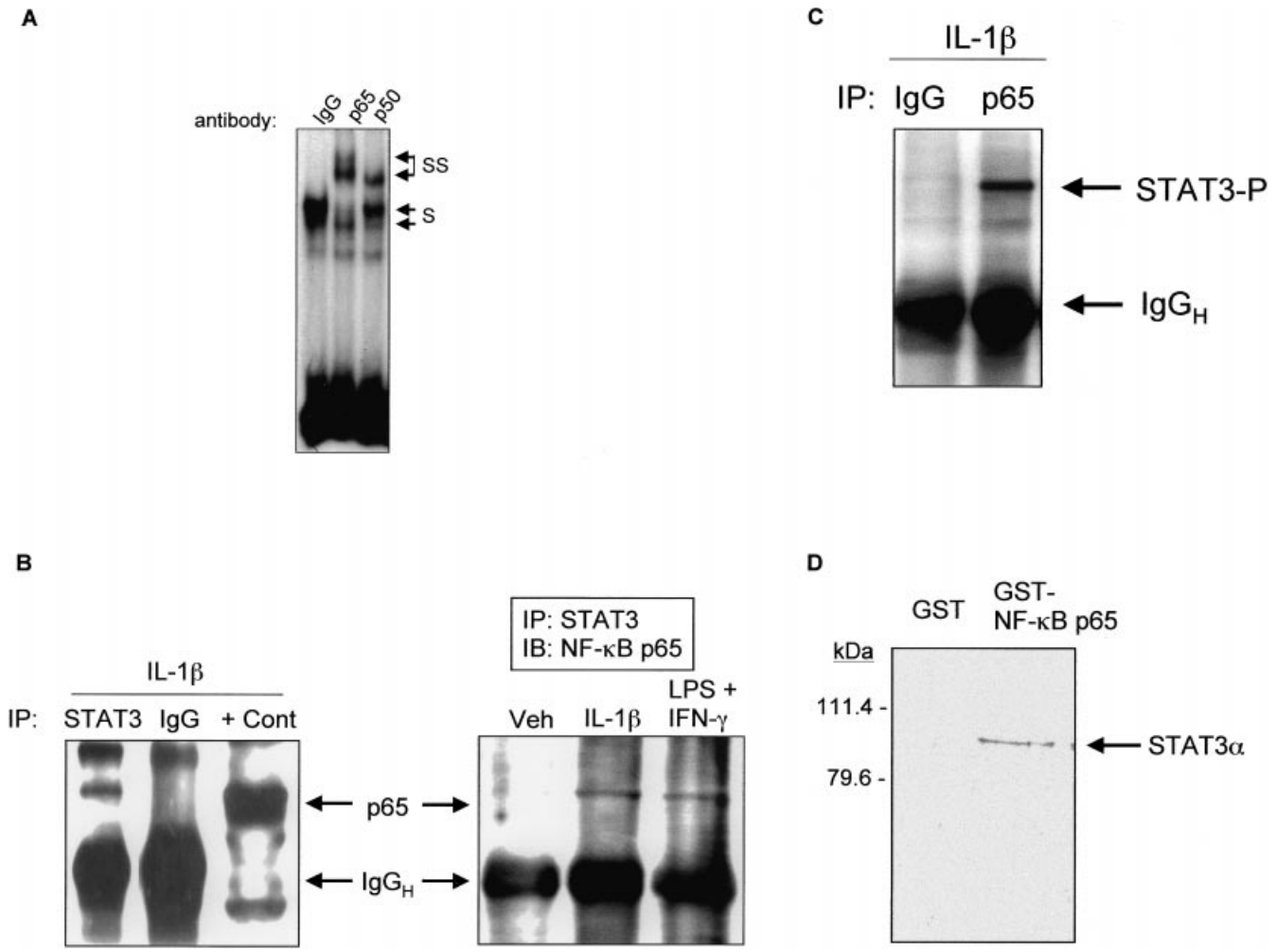

Figure 2 STAT3 interacts with NF-κB p65

(**A**) Supershift experiments were performed in which nuclear extracts from control and IL-1β-treated cells were incubated with the ^{32}P-labelled STAT3 oligomer and antibody specific for NF-κB p65 or p50 or non-immune serum. Gel shifts (S) and supershifts (SS) are indicated. (**B**) Left-hand panel: co-immunoprecipitation of STAT3 and NF-κB p65 from IL-1β-treated mesangial cells. Whole-cell extracts were immunoprecipitated (IP) with polyclonal antibodies directed against STAT3 or non-immune IgG, separated by SDS/PAGE, blotted and probed with polyclonal antibodies directed against NF-κB p65. IgG$_H$ represents IgG heavy chain. An aliquot of nuclear extract was included as a positive control (+Cont). The positions of p65 and of IgG$_H$ are indicated by the arrows. Data are representative of three independent experiments. Right-hand panel: co-immunoprecipitation of STAT3 and NF-κB p65 was performed as described but nuclear extracts were prepared from mesangial cells treated with vehicle (Veh), IL-1β or LPS + IFN-γ ($n = 3$). (**C**) Co-immunoprecipitation of STAT3 and NF-κB p65 from IL-1β-treated mesangial cells. Whole-cell extracts were immunoprecipitated with polyclonal antibodies directed against NF-κB p65 or non-immune IgG, immunoblots were prepared and probed with polyclonal antibodies against Tyr^{705}-phosphorylated STAT3. Data from a representative autoradiograph ($n = 3$) are shown. (**D**) GST-NF-κB p65 or GST alone were incubated with *in vitro*-translated ^{35}S-labelled STAT3α. The GST-NF-κB p65 or GST and any associated protein were isolated with glutathione–Sepharose 4B beads, eluted and analysed by SDS/PAGE and autoradiography. The molecular-mass markers are indicated on the left. Data from a representative autoradiograph ($n = 3$) are shown.

Immunoprecipitation

Stimulated cells were harvested and lysed in RIPA buffer (PBS containing 0.1 % SDS, 0.5 % sodium deoxycholate, 1 % Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF and 3 % protease inhibitor cocktail). These lysates, or nuclear extracts prepared as above, were then precleared by incubating with 20 μl/ml Protein A/G–agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C. After brief centrifugation, the supernatant was added to the indicated primary antibody or control IgG in RIPA buffer (for cell lysates) or nuclear extract buffer (for nuclear extracts) overnight at 4 °C, followed by the addition of 20 μl of Protein A/G–agarose beads. Immunoprecipitates were washed four times in RIPA buffer (for cell lysates) or nuclear extract buffer (for nuclear extracts), resuspended in SDS sample buffer, boiled for 5 min and analysed by SDS/PAGE (8 % gels). Proteins were electrophoretically transferred to PVDF membranes and subjected to Western-blot analysis using the indicated antibodies.

In vitro translation

STAT3α was transcribed and translated from pSG5-STAT3α in the presence of [^{35}S]methionine using T7 RNA polymerase and the TNT Quick-coupled Transcription/Translation Systems kit (Promega) by methods described previously [28].

GST pull-down assays

A GST fusion protein constructed to contain full-length NF-κB p65 was purified from sonicates of isopropyl β-D-thiogalactoside-induced DH5α bacterial cells according to the manufacturer's

© 2002 Biochemical Society

instructions (Amersham Biosciences) and incubated with 50 μl of glutathione–Sepharose 4B beads for 1 h at 4 °C. After centrifugation, the pellet was collected and resuspended in lysis buffer (PBS containing protease inhibitor cocktail). For the *in vitro* binding reaction, 20 μl of purified GST or GST-NF-κB p65 ($\approx$ 4 μg) was incubated in protein binding buffer (20 mM Tris, pH 8.0, 150 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 0.2 % Nonidet P-40 and 10 % glycerol) with 10 μl of [^{35}S]methionine-labelled full-length STAT3α translation product at 4 °C overnight. The samples were then washed four times in binding buffer and boiled in SDS sample buffer, and analysed by SDS/PAGE gel and autoradiography.

Nitrite assays

Mesangial cells, wild-type or transfected, were seeded in 96-well plates and stimulated with IL-1β or LPS + IFN-γ for 24 h. The medium was then collected and the nitrite concentration determined with the Griess Reagent System (Promega) according to the manufacturer's protocol.

Data analysis

Quantitative data are presented as means $\pm$ S.E.M. and were analysed by ANOVA. Significance was assigned at $P < 0.05$.

RESULTS

LPS + IFN-γ and IL-1β promote STAT3 phosphorylation and DNA-binding activity in mesangial cells

Time-course studies of Western blots demonstrated that LPS + IFN-γ induced tyrosine phosphorylation of STAT3 within 15 min of addition (Figure 1A). IL-1β-treated cells exhibited a similar pattern of STAT3 phosphorylation (results not shown). EMSAs with double-stranded oligonucleotides containing a STAT3 consensus sequence and nuclear extracts prepared from control and IL-1β- or LPS + IFN-γ-treated mesangial cells demonstrated that STAT3 DNA-binding activity was induced by these stimuli. As seen in Figure 1(B), major gel shift complexes (complexes I and II) were evident in nuclear extracts prepared from both control and, to a far greater extent, IL-1β-treated cells. The approximate abundance of complex I was consistently much greater in IL-1β-treated cells compared with the controls ($n = 4$). Sequence specificity of the protein–DNA complex was verified in competition experiments: the gel shift band was scarcely evident in the presence of a 50-fold molar excess of unlabelled STAT-binding element oligomers but was apparent when a 50-fold molar excess of unlabelled AP-1 site oligomers were included in the reaction (Figure 1B). LPS + IFN-γ induced similar STAT3 DNA-binding activity (results not shown).

Supershift assays demonstrated that STAT3 proteins contributed to the gel shift complex. Nuclear extracts from IL-1β-treated mesangial cells were preincubated with anti-STAT3 antibody or non-immune serum before reaction with the ^{32}P-labelled oligomers containing the STAT3-binding element probe. The STAT3 antibody partially supershifted the complexes (Figure 1C). STAT1 antibody or non-immune IgG had no effect (results not shown), indicating that the complex contained STAT3, possibly complexed with other proteins.

STAT3 complexes with NF-κB p50 and p65 in activated mesangial cells

Since the STAT3 antibody only partially supershifted the STAT3 DNA–protein complex, we sought to determine whether NF-κB proteins, known to be important for iNOS activation, participated in the complex. Indeed, antibodies against NF-κB p50 and p65 partially supershifted the STAT3 DNA–protein complex (Figure 2A). To determine whether STAT3 and NF-κB p50 and p65 were linked by protein–protein interactions, co-immunoprecipitations were performed reciprocally with polyclonal antibodies directed against STAT3 or polyclonal antibodies directed against NF-κB p50 and p65, and proteins present in the immunoprecipitates were revealed by immunoblotting with the reciprocal antibodies. In both cases, STAT3 and NF-κB p65, and to a far lesser degree p50 (results not shown), were found to co-immunoprecipitate from the nuclear extracts (Figures 2B and 2C). In nuclear extracts of unstimulated cells, no interaction between STAT3 and the NF-κB proteins was observed (Figure 2B). Importantly, these co-immunoprecipitations were carried out using nuclear extracts from non-transfected cells; therefore, the association between STAT3 and NF-κB p50 and p65 does not require overexpression of these proteins. Further evidence for the interaction of these transcription factors was provided from pull-down experiments, which showed that STAT3 could be specifically retained by a GST-NF-κB p65 fusion protein but not by GST alone (see Figure 2D).

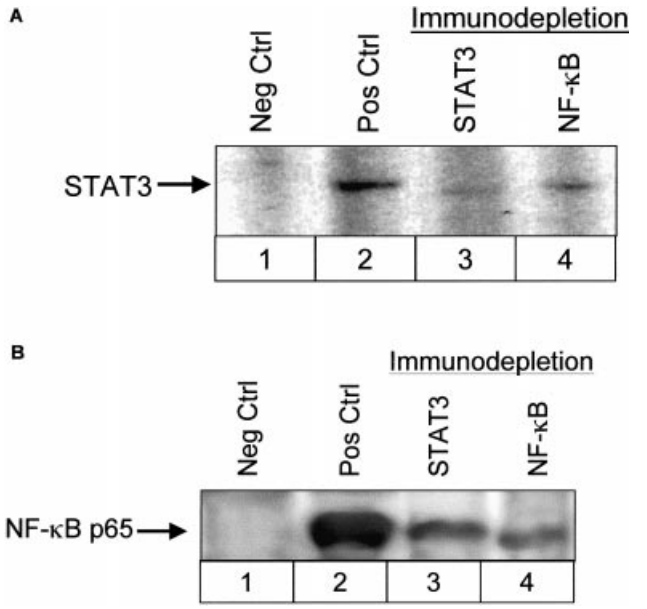

Figure 3 Immunodepletion experiments to estimate the fraction of interacting STAT3 and NF-κB p65

(**A**) Mesangial cells were stimulated with IL-1β for 15 min. Nuclear extracts were prepared, and extracts were either left untreated (lane 2) or sequentially immunodepleted by three rounds of immunoprecipitation with polyclonal antibodies directed against STAT3 (lane 3) or NF-κB p65 (lane 4). A non-immune rabbit serum was used as a negative control (Neg Ctrl, lane 1), while immunoprecipitation without prior immunodepletion served as the positive control (Pos Ctrl, lane 2). Following depletion, supernatants were immunoprecipitated with polyclonal antibodies directed against STAT3 proteins, separated by SDS/PAGE, transferred to a nitrocellulose filter and probed with polyclonal antibodies directed against STAT3 proteins ($n = 3$). Three rounds of immunodepletion with NF-κB p65 antibodies (lane 4) removed the majority of STAT3 proteins from lysates of induced mesangial cells, indicating that a substantial fraction of STAT3 proteins is associated with NF-κB. (**B**) Similar experiment as in (**A**) except that the immunoprecipitations were followed by immunoblotting with anti-NF-κB p65 antibodies. Three rounds of immunodepletion with STAT3 antibodies (lane 3) removed the majority of NF-κB p65 protein from lysates of induced mesangial cells, again indicating that a substantial fraction of STAT3 proteins is associated with NF-κB ($n = 3$).

© 2002 Biochemical Society

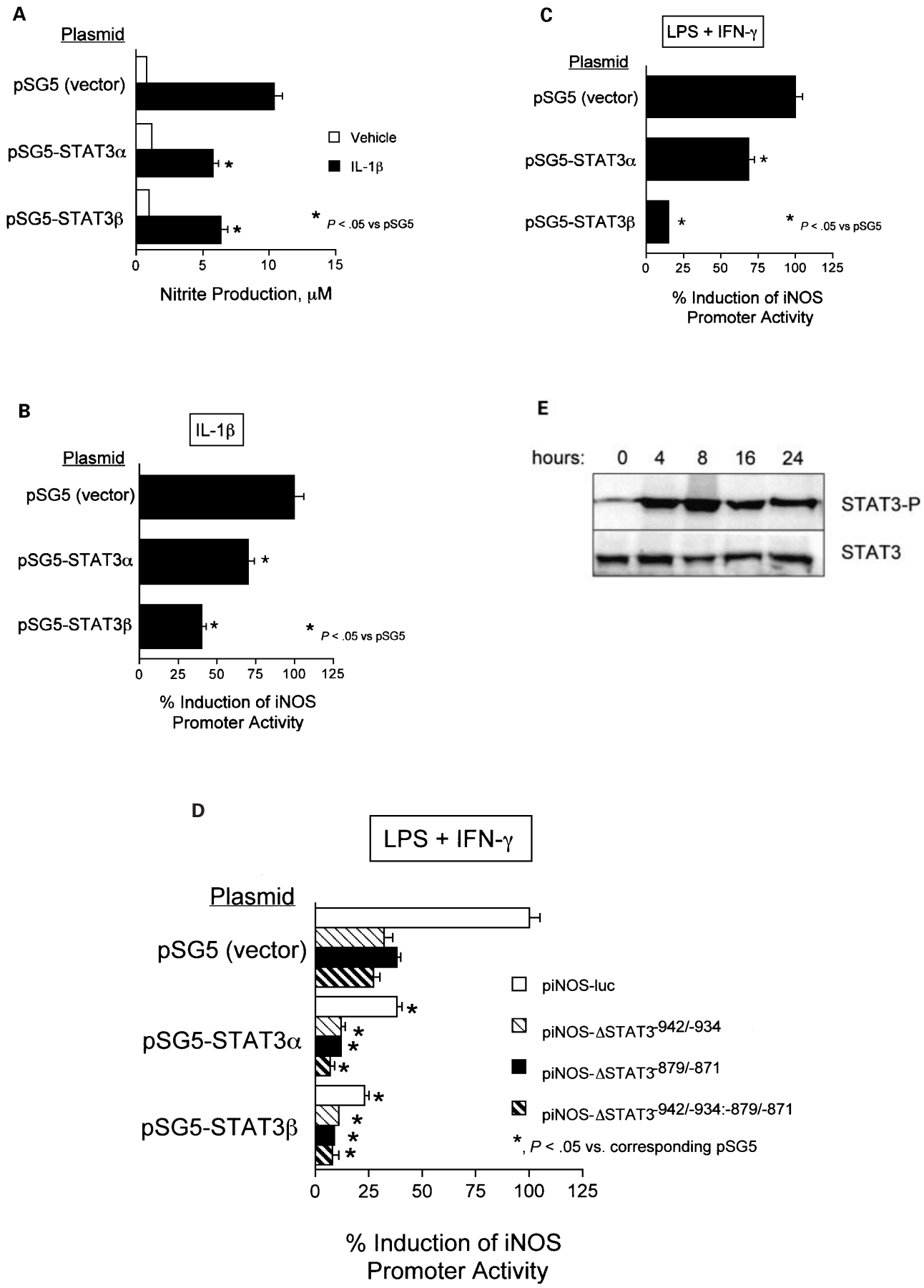

Figure 4 For caption, see facing page

© 2002 Biochemical Society

To estimate the relative amount of complexed NF-κB and STAT3 compared with the total pool of the proteins, proteins were depleted from nuclear extracts by three sequential rounds of immunoprecipitations with NF-κB p65 antibody. Depleted lysates were then precipitated and blotted with antibodies to STAT3 to estimate the residual level of STAT3 that remained unassociated with NF-κB protein. As shown in Figure 3(A), three sequential rounds of immunodepletion with NF-κB antibodies removed the majority of STAT3 proteins from lysates, indicating that a substantial fraction of STAT3 protein is associated with NF-κB in mesangial cells following IL-1β or LPS + IFN-γ stimulation. No depletion was observed when a non-immune serum was used (Figure 3A, positive control). In the reciprocal experiment, three sequential rounds of immunoprecipitation with STAT3 antibody were performed, and the depleted lysates were subsequently precipitated and immunoblotted with antibodies to NF-κB p65 (Figure 3B). This immunodepletion with STAT3 antibodies removed the majority of NF-κB p65 proteins from the lysates, supporting the conclusion that a large pool of NF-κB p65 and STAT3 interact.

In the aggregate, these experiments indicate that the STAT3-specific DNA–protein complex primarily contains STAT3 and NF-κB p65 and that these proteins physically interact.

Overexpression of STAT3 inhibits iNOS induction

Previous work in vascular smooth muscle cells demonstrated that immunodepletion of STAT3 augmented iNOS induction, suggesting that STAT3 exerts an inhibitory effect on iNOS gene expression [14]. We hypothesized that STAT3 would similarly inhibit iNOS induction in mesangial cells and if so, sought to determine the specific mechanisms for this effect. We further hypothesized that the physical interaction of STAT3 and NF-κB p65 (Figure 2) might influence iNOS transcriptional activity. Accordingly, mesangial cells were co-transfected with empty expression vector (pSG5) or the expression plasmids pSG5-STAT3α or pSG5-STAT3β. This achieved high-level expression of STAT3, which upon induction with LPS + IFN-γ or IL-1β resulted in abundant expression of phosphorylated STAT3 (Figure 4E and results not shown). Mesangial cells co-transfected with pSG5-STAT3α or pSG5-STAT3β produced significantly less (70 % and 50 %, respectively) nitrite in response to IL-1β stimulation compared with cells transfected with pSG5 (Figure 4A). Since iNOS is the only NOS isoform expressed in these cells, these data indicate that overexpressed STAT3 suppresses endogenous iNOS-mediated NO generation. This inhibitory effect on NO production was in large part transcriptionally mediated, since mesangial cells co-transfected with the iNOS promoter construct piNOS-luc and pSG5-STAT3α or pSG5-STAT3β exhibited IL-1β-stimulated iNOS promoter activity that was $\approx$ 40 % lower than that of the vector-transfected controls (Figure 4B). Similar *trans*-repression of iNOS promoter activity was observed following LPS + IFN-γ induction (Figure 4C).

To determine whether these effects could be generalized to other iNOS-expressing cell types, we tested the ability of STAT3 to inhibit iNOS induction in LPS + IFN-γ-treated RAW 264.7 cells, which are known to have robust iNOS gene transcription in response to these stimuli. As occurred in mesangial cells, overexpression of STAT3α or STAT3β resulted in a respective 67 ± 7 % and 89 ± 4 % ($P < 0.05$, $n = 3$) *trans*-repression of piNOS-luc promoter activity in RAW 264.7 cells. Thus the suppressive effect of STAT3 on iNOS induction is not restricted to mesangial cells.

We explored two potential mechanisms, direct and indirect, for the ability of STAT3 to *trans*-repress the iNOS promoter in mesangial cells. First, we tested whether STAT3 *trans*-represses the iNOS promoter by binding to GAS elements in the iNOS promoter. As seen in Figure 4(D), mutation of the GAS consensus sites in the iNOS promoter resulted in a $\approx$ 65 % reduction in maximal IL-1β induction, in agreement with previous work in LPS + IFN-γ-treated RAW 264.7 cells showing that STAT1 *trans*-activates the iNOS promoter via the GAS element [13]. However, although inducible promoter activity levels were reduced, mutation of the GAS sites did not relieve the STAT3-dependent inhibition of iNOS promoter activity in mesangial cells (Figure 4D). These results indicate that STAT3 binding to its cognate GAS elements is not required for the inhibitory actions of STAT3 on iNOS gene transcription.

Since NF-κB is known to be a potent *trans*-activator of the iNOS promoter [12], and since we demonstrated a physical association of STAT3 and NF-κB p50 and p65, we hypothesized that STAT3 might exert its inhibitory actions on iNOS transcription in part by limiting the availability of NF-κB to sites on the iNOS promoter. Consistent with this hypothesis, overexpression of STAT3 dramatically inhibited the activity of an NF-κB-dependent promoter that lacks STAT-binding elements (Figure 5A). This effect occurred without a discernable difference in NF-κB DNA-binding activity (Figure 5B). Furthermore, gel shift assays with the NF-κBu probe demonstrated that overexpression of STAT3α or STAT3β did not significantly alter the NF-κB DNA-binding activity in these cells (Figure 5B). Thus, although STAT3 interacts with NF-κB, it does not measurably modify NF-κB DNA binding. Collectively, these results indicated that STAT3 serves as an indirect inhibitor of cytokine-induced iNOS transcription in mesangial cells by serving as a dominant-negative inhibitor of NF-κB under these conditions.

DISCUSSION

Resting mesangial cells produce low basal levels of inflammatory mediators, including NO, but soluble factors produced by inflammatory cells that invade the glomerulus or by circulating factors can activate iNOS and stimulate NO production. Because of the potent biological actions of NO, considerable attention has been placed on identifying the mechanisms that activate and limit iNOS gene expression. In this report, we describe a new

Figure 4 Overexpression of STAT3 inhibits induction of endogenous NO generation and the activity of the murine iNOS promoter

(**A**) Mesangial cells were transiently co-transfected with the STAT expression plasmids pSG5-STAT3α or pSG5-STAT3β, or the parent vector pSG5. After transfection, the cells were treated with vehicle or IL-1β, and the nitrite production in the supernatant was measured by the Greiss reagent. (**B, C**) Mesangial cells were transiently co-transfected with the STAT expression plasmids pSG5-STAT3α or pSG5-STAT3β, or the parent vector pSG5 and piNOS-luc, containing the wild-type iNOS promoter, as well as with a *Renilla* luciferase expression plasmid. After transfection, the cells were treated with vehicle together with IL-1β (**B**) or LPS + IFN-γ (**C**), and the luciferase activity in cell lysates was measured ($n = 5$). (**D**) Mesangial cells were transiently co-transfected with the STAT expression plasmids pSG5-STAT3α or pSG5-STAT3β, or the parent vector pSG5 and iNOS promoter constructs piNOS-luc, containing the wild-type iNOS promoter, piNOS-ΔSTAT3$^{-942/-934}$-luc or piNOS-ΔSTAT3$^{-879/-871}$-luc, or both, which bear mutations of the $-942/-934$ and $-879/-871$ GAS elements, respectively, of the iNOS promoter, as well as with a *Renilla* luciferase expression plasmid. After transfection, the cells were treated with vehicle or LPS + IFN-γ, and the luciferase activity in cell lysates was measured. (**E**) Mesangial cells were transiently transfected with the STAT3α expression plasmid pSG5-STAT3α transfection, the cells were treated with LPS + IFN-γ for the indicated times, nuclear extracts were harvested and immunoblots prepared and blotted with phospho-Tyr^{705}-STAT3-specific antibody or a polyclonal antibody directed against STAT3 ($n = 3$). Similar high-level expression of phospho-STAT3 after induction was observed when cells were treated with IL-1β or when STAT3β was overexpressed and treated with IL-1β or LPS + IFN-γ (results not shown).

© 2002 Biochemical Society

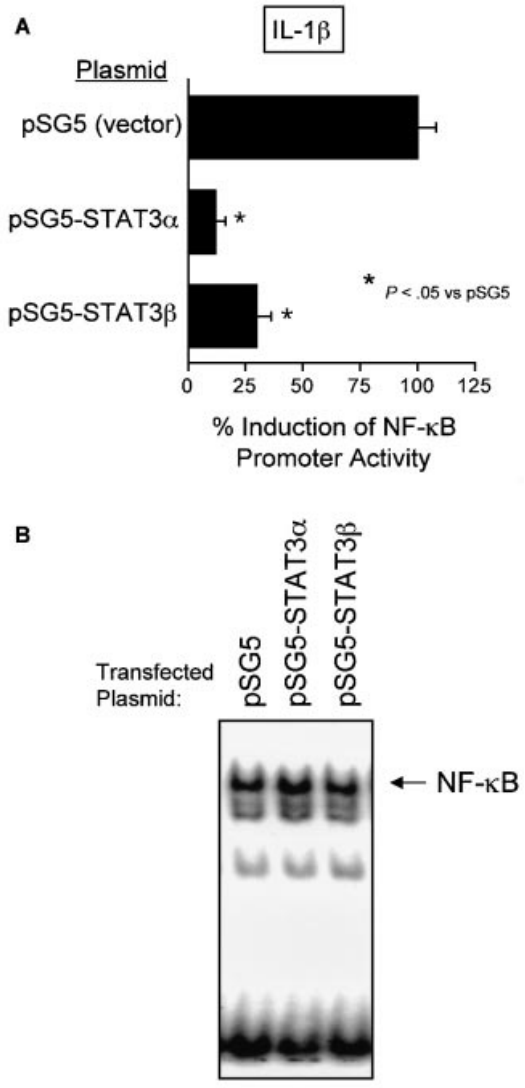

Figure 5 (A) STAT3 suppresses the transcriptional activity of an NF-κB element reporter construct and (B) STAT3 overexpression does not alter NF-κB DNA-binding activity in IL-1β-treated mesangial cells

(**A**) Mesangial cells were co-transfected with a vector expressing a luciferase reporter gene containing three copies of an NF-κB consensus binding site linked to a minimal simian virus 40 promoter together with empty vector or vector encoding STAT3α or STAT3β, as well as with a *Renilla* luciferase expression vector. Following transfection, cells were treated with vehicle or IL-1β for 24 h. Cell extracts were then prepared and processed to measure luciferase activity ($n = 6$). (**B**) Mesangial cells were transfected with STAT expression plasmids pSG5-STAT3α or pSG5-STAT3β or the parent vector pSG5. Then, 24 h later, the cells were treated with IL-1β for 15 min, nuclear extracts were prepared, and EMSA with an NF-κB consensus-binding-site probe was performed ($n = 3$).

pathway for the down-regulation of iNOS induction and a novel biological role for STAT3 in the counter-regulation of iNOS activation and probably the activation of other NF-κB-responsive genes. We demonstrate a functional and direct association between STAT3 and NF-κB p65 transcription factors that serves to repress induction of the murine iNOS gene in activated mesangial cells. We show that STAT3 inhibits cytokine induction of endogenous NO generation (Figure 4A) and NF-κB p65 signalling to the iNOS promoter (Figures 4B and 4C) as well as to an NF-κB promoter construct (Figure 5). This antagonistic action of STAT3 with NF-κB proteins provides the potential to fine-tune the expression of downstream target genes in response to a variety of growth factors and cytokines.

Our study complements and extends the study of Marrero et al. [14], who examined STAT3 and iNOS in vascular smooth muscle cells. In that study, electroporation of neutralizing antibodies against STAT1 and STAT3 increased iNOS protein abundance. However, the specific mechanism for the inhibitory effect was not established. Our results indicate that STAT3 is activated in response to IL-1β or LPS + IFN-γ and that overexpression of STAT3α or STAT3β inhibits the induction of NO production (Figure 4A) and the activity of the iNOS promoter (Figures 4B and 4C). The ability of STAT3 to inhibit iNOS promoter constructs was evident even when the −942/−934 and −879/−871 GAS sites were mutated (Figure 4D). In addition, STAT3 overexpression inhibited the activity of a heterologous promoter containing a three-repeat palindrome of NF-κB elements and no GAS elements (Figure 5). The fact that the inhibitory effect does not require STAT3 to bind a cognate DNA element to inhibit iNOS promoter activity suggests that the inhibition is mediated by protein–protein interaction with NF-κB or by competing for a factor(s) that is necessary for NF-κB induction of the iNOS promoter. In the case of STAT5b, for example, the co-activator p300/CBP reversed, by competition, STAT5b inhibition of a NF-κB-thymidine kinase promoter construct [22]. These results further support our hypothesis that STAT3-mediated inhibition of NF-κB signalling is not mediated by STAT3–DNA interactions but by protein–protein interactions. Indeed, supershift assays (Figure 2A), co-immunoprecipitation experiments (Figure 2B) and GST pull-down experiments (Figure 2C) showed that the two transcription factors interact *in vivo* and *in vitro*. The pull-down experiments suggest that the interaction *in vitro* is direct and does not require accessory proteins. Since STAT3 can inhibit iNOS promoter function indirectly, without the need for DNA binding in this context, the function of the STAT3 DNA-binding activity that we observed (Figure 1B) remains unclear. Since phosphorylated STAT3 was negligible under basal conditions on immunoblots of nuclear extracts from mesangial cells (Figure 1A), and since STAT3 phosphorylation is generally believed to be required for translocation to the nucleus, it may be that other constitutively expressed transcription factors complex with the STAT3-binding element at low levels under basal conditions.

In keeping with our results, recent studies have shown that the activities of STAT factors can be modulated by their interactions with other DNA-binding proteins and non-DNA-binding proteins, such as co-activators. STAT3 has been shown to interact with c-Jun [29], c-Fos and the CREB-binding protein co-activators [30]. *In vitro* pull-down assays identified a segment of STAT3 from residues ≈ 130–358 which binds to the C-terminus of c-Jun [29]. The domain(s) that modulate(s) the interaction between STAT3 and NF-κB proteins remain(s) to be identified. It is unlikely that this interaction involves the DNA-binding domain of NF-κB, since STAT3 overexpression did not significantly interfere with DNA binding. Several regions of STAT proteins have been previously implicated in transcriptional regulation: inactivation by tyrosine phosphatases requires the N-terminal domain, whereas ubiquitination is dependent on the C-terminal part of STAT proteins. The N-terminal region of STAT proteins is involved in dimer–dimer interactions leading to co-operative DNA binding [31]. The STAT3 C-terminus also functions in protein–protein interactions and was recently demonstrated to be capable of recruiting p300/CBP [32]. STAT3β appeared to be more effective at inhibiting IL-1β- or LPS + IFN-γ-induced iNOS promoter activity in mesangial cells. In contrast, STAT3α appeared to be more effective than STAT3β in inhibiting

© 2002 Biochemical Society

NF-κB promoter activity in these same cells, and in inhibiting iNOS promoter activity in RAW 264.7 cells. Since minor differences in transfection efficiency and expression level under these different circumstances may have occurred, however, it is difficult to state with certainty that one of the isoforms is more potent in inhibiting iNOS promoter activity or NF-κB-driven promoter activity than the other.

Other mechanisms for down-regulation of iNOS transcription have been reported. Dexamethasone inhibits cytokine-induced iNOS mRNA in part by limiting nuclear NF-κB but also by competition for the co-activator CBP/p300 [33]. Peroxisome-proliferator-activated receptor γ inhibits iNOS transcription in part by targeting the CBP/SRC-1 co-activator complex and antagonizing the activities of STAT1, NF-κB and AP-1 [34]. Our findings add STAT3 to the short list of regulatory factors limiting iNOS biosynthesis and high-output NO generation. They distinguish STAT3α and STAT3β as novel transcriptional inhibitors of the iNOS gene in mesangial cells and identify a previously unrecognized interaction between STAT3 and NF-κB proteins. This novel mechanism may serve to control levels of the iNOS enzyme so as to avoid cytotoxic effects of NO to the host or bystander cells.

This work was supported by National Institutes of Health grants RO1 DK50745 and P50 GM38529 and the Department of Defense DREAMS grant to B.C.K. We thank Dr David Tweardy (Baylor College of Medicine) and Dr Bharat Aggarwal (University of Texas M.D. Anderson Cancer Center) for their generous gifts of plasmids used in this paper, and Sandra Higham for expert technical assistance.

REFERENCES

1 Raij, L. and Baylis, C. (1995) Glomerular actions of nitric oxide. Kidney Int. **48**, 20–32
2 Furusu, A., Miyazaki, M., Abe, K., Tsukasaki, S., Shioshita, K., Sasaki, O., Miyazaki, K., Ozono, Y., Koji, T., Harada, T. et al. (1998) Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. Kidney Int. **53**, 1760–1768
3 Narita, I., Border, W. A., Ketteler, M. and Noble, N. A. (1995) Nitric oxide mediates immunologic injury to kidney mesangium in experimental glomerulonephritis. Lab. Invest. **72**, 17–24
4 Kone, B. C. (1997) Nitric oxide in renal health and disease. Am. J. Kidney Dis. **30**, 311–333
5 Eberhardt, W., Pluss, C., Hummel, R. and Pfeilschifter, J. (1998) Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. J. Immunol. **160**, 4961–4969
6 Sharma, K., Danoff, T. M., DePiero, A. and Ziyadeh, F. N. (1995) Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: possible mediation via protein kinase C. Biochem. Biophys. Res. Commun. **207**, 80–88
7 Guan, Z., Baier, L. D. and Morrison, A. R. (1997) p38 mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E2 biosynthesis stimulated by interleukin-1β. J. Biol. Chem. **272**, 8083–8089
8 Beck, K. F. and Sterzel, R. B. (1996) Cloning and sequencing of the proximal promoter of the rat iNOS gene activation of NF-κB is not sufficient for transcription of the iNOS gene in rat mesangial cells. FEBS Lett. **394**, 263–267
9 Pfeilschifter, J. and Vosbeck, K. (1991) Transforming growth factor β2 inhibits interleukin 1β and tumour necrosis factor α induction of nitric oxide synthase in rat renal mesangial cells. Biochem. Biophys. Res. Commun. **175**, 372–379
10 Saura, M., Martinez-Dalmau, R., Minty, A., Perez-Sala, D. and Lamas, S. (1996) Interleukin-13 inhibits inducible nitric oxide synthase expression in human mesangial cells. Biochem. J. **313**, 641–646
11 Nakashima, O., Terada, Y., Inoshita, S., Kuwahara, M., Sasaki, S. and Marumo, F. (1999) Inducible nitric oxide synthase can be induced in the absence of active nuclear factor-κB in rat mesangial cells: involvement of the Janus kinase 2 signaling pathway. J. Am. Soc. Nephrol. **10**, 721–729
12 Xie, Q. W., Kashiwabara, Y. and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J. Biol. Chem. **269**, 4705–4708
13 Gao, J., Morrison, D. C., Parmely, T. J., Russell, S. W. and Murphy, W. J. (1997) An interferon-γ-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-γ and lipopolysaccharide. J. Biol. Chem. **272**, 1226–1230
14 Marrero, M. B., Venema, V. J., He, H., Caldwell, R. B. and Venema, R. C. (1998) Inhibition by the JAK/STAT pathway of IFNγ- and LPS-stimulated nitric oxide synthase induction in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. **252**, 508–512
15 Bromberg, J. and Darnell, Jr, J. E. (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene **19**, 2468–2473
16 Stocklin, E., Wissler, M., Gouilleux, F. and Groner, B. (1996) Functional interactions between Stat5 and the glucocorticoid receptor. Nature (London) **383**, 726–728
17 Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K. and Rosenfeld, M. G. (1998) Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science **279**, 703–707
18 Caldenhoven, E., van Dijk, T. B., Solari, R., Armstrong, J., Raaijmakers, J. A., Lammers, J. W., Koenderman, L. and de Groot, R. P. (1996) STAT3β, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. J. Biol. Chem. **271**, 13221–13227
19 Ohmori, Y., Schreiber, R. D. and Hamilton, T. A. (1997) Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. J. Biol. Chem. **272**, 14899–14907
20 Shen, C. H. and Stavnezer, J. (1998) Interaction of Stat6 and NF-κB: direct association and synergistic activation of interleukin-4-induced transcription. Mol. Cell. Biol. **18**, 3395–3404
21 Pine, R. (1997) Convergence of TNFα and IFNγ signalling pathways through synergistic induction of IRF-1/ISGF-2 is mediated by a composite GAS/κB promoter element. Nucleic Acids Res. **25**, 4346–4354
22 Luo, G. and Yu-Lee, L. (2000) Stat5β inhibits NF-κB-mediated signaling. Mol. Endocrinol. **14**, 114–123
23 Pfitzner, E., Jahne, R., Wissler, M., Stoecklin, E. and Groner, B. (1998) p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. Mol. Endocrinol. **12**, 1582–1593
24 Darnay, B. G., Ni, J., Moore, P. A. and Aggarwal, B. B. (1999) Activation of NF-κB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-κB-inducing kinase. Identification of a novel TRAF6 interaction motif. J. Biol. Chem. **274**, 7724–7731
25 Gupta, A. K. and Kone, B. C. (1999) CCAAT/enhancer binding protein-β trans-activates murine nitric oxide synthase 2 gene in an MTAL cell line. Am. J. Physiol. **276**, F599–F605
26 Gupta, A. K., Diaz, R. A., Higham, S. and Kone, B. C. (2000) α-MSH inhibits induction of C/EBPβ-DNA binding activity and NOS2 gene transcription in macrophages. Kidney Int. **57**, 2239–2248
27 Yu, C. L., Meyer, D. J., Campbell, G. S., Larner, A. C., Carter-Su, C., Schwartz, J. and Jove, R. (1995) Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science **269**, 81–83
28 Kuncewicz, T., Balakrishnan, P., Snuggs, M. B. and Kone, B. C. (2001) Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. Am. J. Physiol. Renal Physiol. **281**, F326–F336
29 Zhang, X., Wrzeszczynska, M. H., Horvath, C. M. and Darnell, Jr, J. E. (1999) Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. Mol. Cell. Biol. **19**, 7138–7146
30 Coqueret, O. and Gascan, H. (2000) Functional interaction of STAT3 transcription factor with the cell cycle inhibitor p21WAF1/CIP1/SDI1. J. Biol. Chem. **275**, 18794–18800
31 Decker, T. and Kovarik, P. (1999) Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. Cell. Mol. Life Sci. **55**, 1535–1546
32 Paulson, M., Pisharody, S., Pan, L., Guadagno, S., Mui, A. L. and Levy, D. E. (1999) Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. J. Biol. Chem. **274**, 25343–25349
33 McKay, L. I. and Cidlowski, J. A. (2000) CBP (CREB binding protein) integrates NF-κB (nuclear factor-κB) and glucocorticoid receptor physical interactions and antagonism. Mol. Endocrinol. **14**, 1222–1234
34 Li, M., Pascual, G. and Glass, C. K. (2000) Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. Mol. Cell. Biol. **20**, 4699–4707

Received 12 April 2002/29 May 2002; accepted 11 June 2002
Published as BJ Immediate Publication 11 June 2002, DOI 10.1042/BJ20020588

© 2002 Biochemical Society