

The STAT3 DNA-Binding Domain Mediates Interaction with NF- κ B p65 and Inducible Nitric Oxide Synthase Transrepression in Mesangial Cells

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Abstract. Signal transducer and activator of transcription 3 (STAT3) and nuclear factor κ B (NF- κ B) are important transcription factors involved in glomerulonephritis and other inflammatory processes, including transcription of the inducible nitric oxide synthase (iNOS) gene. The ability of STAT3 to interact physically with NF- κ B p65 in glomerular mesangial cells and thereby to inhibit NF- κ B-mediated transactivation of the iNOS gene was demonstrated previously. STAT3 is a modular protein with several structurally and functionally defined domains. For defining STAT3 domains that interact with NF- κ B p65, 35 S-labeled proteins that corresponded to each STAT3 α domain were synthesized, and their ability to bind specifically a GST-NF- κ B p65 fusion protein in GST pulldown assays was tested. The coiled-coil and DNA-binding domains were specifically retained by GST-NF- κ B p65, whereas the N-terminal, linker domain, Src homology 2 domain, and tran-

scriptional activation domain failed to interact with NF- κ B p65. Deletion of the region L³⁵⁸ through I³⁶⁹ of the STAT3 DNA-binding domain greatly reduced binding to GST-NF- κ B p65. Alanine substitution mutations at four highly conserved residues—L³⁵⁸, N³⁵⁹, K³⁶³, and V³⁶⁶—in this region greatly abrogated the ability of STAT3 to bind NF- κ B p65. Moreover, in contrast to the transrepression afforded by wild-type STAT3 α , a STAT3 α construct harboring these mutations, failed to suppress endogenous NO production and to transrepress iNOS promoter-reporter and κ B element-reporter constructs in IL- β -stimulated mesangial cells. These data reveal a novel role for the DNA-binding domain in the physical and functional coupling of STAT3 to NF- κ B p65 that is important for regulating the transcriptional activity of iNOS and likely other NF- κ B p65 responsive genes that are important for mesangial cell responses.

Signal transducers and activators of transcription (STAT) are a seven-member family of latent transcription factors that mediate cellular responses to diverse cytokines, hormones, and growth factors. When activated, STAT factors can stimulate or inhibit gene transcription, depending, in part, on their interaction with other transcription factors (1, 2) and/or coactivators/corepressors (3). The STAT family participates in the regulation of genes that are involved in an array of essential cell processes, including the acute-phase response, inflammation, cell growth, and differentiation. Of these family members, STAT3 is principally expressed in the kidney, liver, and spleen. It is alternatively spliced to yield two isoforms, termed STAT3 α and STAT3 β . STAT3 β differs from STAT3 α only in that it lacks the 55 C-terminal amino acid residues of STAT3 α , and it has seven additional amino acid residues at its C-terminus (4). In glomerular mesangial cells, STAT3 partici-

pates in thrombin-induced expression of the *TIMP-1* gene (5), the response to high glucose and angiotensin II (6, 7), and in GAS6-mediated cellular proliferation (8). Nuclear factor κ B (NF- κ B) is an inducible transcription factor that plays a role in the expression in inducible nitric oxide synthase (iNOS) and various other genes involved in immune and inflammatory responses in numerous cell types, including mesangial cells. Although activated by different pathways, activated STAT and NF- κ B translocate into the nucleus and function either individually or cooperatively in regulating the expression of target genes (9–12).

STAT share a highly conserved, modular structure containing multiple functional domains. The crystal structures of STAT3 β lacking the NH₂-terminal domain (N-domain) show several conserved domains: a coiled-coil domain, a DNA-binding domain, a linker domain, and a Src homology 2 (SH2) domain (Figure 1). Forty to 50 amino acids at the carboxyl termini of STAT that are absent in the crystal structures compose the transcriptional activation domain that may also be involved in the protease-dependent turnover of STAT5 (13). The N-domain contains the NH₂-terminal 125 amino acids, and it mediates dimer–dimer interactions that lead to cooperative DNA binding (14, 15) and nuclear translocation (16). The coiled-coil domain (amino acids 135 to 315) is essential for cytokine- and growth factor-stimulated recruitment of STAT3

Received August 11, 2003. Accepted December 7, 2003.

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1046-6673/1503-0585

Journal of the American Society of Nephrology

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DOI: 10.1097/01.ASN.0000114556.19556.F9

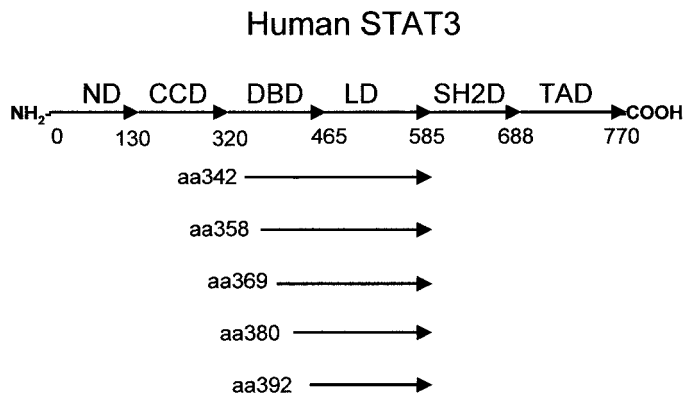


Figure 1. Schematic diagrams of the structural domains and the deletion mutants of STAT3. Abbreviations of each domain of STAT3 are indicated on the figures. ND, N-terminal domain; CCD, coiled-coil domain; DBD, DNA-binding domain; LD, linker domain; SH2D, SH2 domain; TAD, transcription activation domain.

to the receptor, dimer formation, nuclear translocation, and DNA binding (17, 18). The DNA-binding domain (amino acids 320 to 480) recognizes members of the GAS family of enhancers and seems to regulate nuclear export (14, 19). The adjacent linker domain (amino acids 480 to 575) ensures the appropriate structure of the DNA-binding motif and may regulate nuclear export in resting cells. The SH2 domain (amino acids 575 to 680), the most highly conserved motif, docks the protein to tyrosine phosphorylated receptor subunits, promotes dimerization, and may also associate with the activating Jak (20). The STAT C-terminus contains an autonomously functioning transcriptional activation domain, absent from spliced isoforms of STAT1, STAT3, and STAT4, and this region also functions in protein–protein interactions (21).

Recently we reported a physical and functional interaction of STAT3 with NF- κ B p65 that inhibits transcriptional activation of the iNOS gene (1). In that study, STAT3 was found to bind to NF- κ B p65 and to inhibit NF- κ B–mediated transactivation of the iNOS gene and endogenous NO production in cultured glomerular mesangial cells by a mechanism(s) that did not impair the ability of NF- κ B to bind its cognate elements in the promoter and that did not require STAT3 itself to bind directly to the promoter. In this report, we extend our studies by using mutational analysis of expressed proteins to show that the DNA-binding domain of STAT3 is necessary and sufficient for NF- κ B p65 interaction and for the consequent transrepression of the iNOS gene and endogenous NO production in mesangial cells. These results also reveal a novel function of the STAT3 DNA-binding domain to mediate co-regulatory interactions with other transcription factors.

Materials and Methods

Cell Culture and Reagents

Mouse mesangial cells (ATCC CRL-1927) were maintained in Ham's F12 plus DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5% FBS. Vehicle or IL-1 β (10 ng/ml) was added to the cells as indicated in the text and figure legends. Mouse recombinant IL-1 β was from R & D Systems

(Minneapolis, MN). A polyclonal antibody recognizing STAT3 (C20) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). A polyclonal antibody recognizing NF- κ B p65 was from Upstate Biotechnology (Lake Placid, NY). Oligonucleotides were custom synthesized by Genosys (The Woodlands, TX). Lipofectamine 2000 reagent was from Invitrogen (Carlsbad, CA). The Dual-Luciferase Reporter Assay System and the luciferase vectors pGL3-Basic and pRL-SV40 were from Promega (Madison, WI). The BCA protein estimation kit was from Pierce Chemical (Rockford, IL). Glutathione-Sepharose 4B beads, pGEX-5X-3, and ECL reagents were from Amersham Pharmacia Biotech (Piscataway, NJ).

Plasmids and Site-Directed Mutagenesis

The STAT3 α expression plasmid pSG5-STAT3 α , which encodes human STAT3 α and was used in our previous study (3), was provided by Dr. David Tweardy (Baylor College of Medicine, Houston, TX). The glutathione S-transferase (GST)–NF- κ B p65 fusion protein encoding full-length NF- κ B p65 was described (1). The NF- κ B reporter construct, p36B(–)(NF- κ B)₃-luc, which contains three tandem copies of the κ B binding element (GGGGACTCTCCC) upstream of the SV40 early promoter sequence and fused to the coding sequence for the luciferase gene (22), was a gift from Dr. Bharat Agarwal (The University of Texas M.D. Anderson Cancer Center). piNOS-luc, containing the proximal 1.8 kb of the mouse iNOS promoter fused to the firefly luciferase gene in the vector pGL3, has been described (1). DNA encoding the specific STAT3 domains (Figure 1) were generated by PCR using STAT3 α as a template and with incorporation of an upstream T7 promoter sequence for *in vitro* transcription. pSG5-STAT3 α ^{ΔDBD}, which harbors alanine substitution mutations at L³⁵⁸, N³⁵⁹, K³⁶³, and V³⁶⁶, was generated from STAT3 α by PCR overlap extension. Inserts generated by PCR were sequenced to verify their authenticity.

Transient Transfections

Mesangial cells were seeded in 24-well plates and grown to 90 to 95% confluence in complete medium without antibiotics and transfected the following day using the LipoFectamine 2000 reagent following the manufacturer's protocol and a total of 1 μ g/well plasmid DNA. The amount of transfected DNA was kept constant by addition of appropriate amounts of the parental empty expression vector. For transrepression promoter-reporter gene experiments, 0.78 μ g of piNOS-luc, p36B(–)(NF- κ B)₃-luc, or promoterless expression vector was co-transfected with 0.2 μ g of pSG5-STAT3 α , pSG5-STAT3 α ^{ΔDBD}, or insertless expression vector pSG5, along with 0.02 μ g of the *Renilla* luciferase expression plasmid pRL-SV40pRL-SV40. Twenty-four hours after transfection, vehicle or IL-1 β (10 ng/ml) was added to the medium. Twenty-four hours later, cell lysates for measurement of firefly and *Renilla* luciferase activities were prepared, and firefly and *Renilla* luciferase activities in 100- μ l lysate samples were measured as described previously in our laboratory (23). For experiments to examine the effects of wild-type and mutated STAT3 on endogenous nitrite production, mesangial cells were transfected with 0.3 μ g of pSG5-STAT3 α , pSG5-STAT3 α ^{ΔDBD}, or insertless expression vector pSG5 and after 24 h were stimulated with vehicle or IL-1 β (10 ng/ml). After an additional 24 h, medium was collected for nitrite determinations by the Greiss reaction as described previously (1). The transfection efficiencies, judged by transfection of pSV- β -galactosidase control vector (Promega) followed by staining with X-gal (5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside) and cell counting, was reproducibly 40 to 50%.

In Vitro Transcription and Translation

STAT3 α was transcribed and translated from pSG5-STAT3 α in the presence of [35 S]methionine using the TNT Quick Coupled Transcription/Translation System Kit (Promega) by methods previously described (1). Using the STAT3 cDNA driven by the T7 promoter sequence that we generated, different domains of STAT3 were similarly transcribed and translated in the presence or absence of [35 S]methionine as indicated in the text and figure legends. Luciferase was transcribed and translated from luciferase SP6/T7 control vector (Promega) using the TNT Quick Coupled Transcription/Translation System. Translation products from each construct were routinely analyzed by SDS-PAGE and autoradiography to estimate abundance and to confirm that the products were of appropriate size and quality.

GST Pulldown Assays

The GST-NF- κ B p65 fusion protein was purified from sonicates of isopropyl β -D-thiogalactoside-induced DH5 α bacterial cells according to the manufacturer's instruction (Amersham Pharmacia Biotech) and incubated with 50 μ l of glutathione-Sepharose 4B beads for 1 h at 4°C. After centrifugation, the pellets were collected and resuspended in lysis buffer (PBS containing protease inhibitor cocktail). For the *in vitro* binding reaction, 20 μ l of purified, bead-bound GST or GST-NF- κ B p65 was incubated in protein-binding buffer (20 mM Tris [pH 8.0], 150 mM KCl, 1 mM EDTA, 4 mM MgCl₂, 0.2% NP-40, 10% glycerol) with 10 μ l of [35 S]methionine-labeled or unlabeled (as indicated in the text and figure legends) translation product of full-length or truncated STAT3 α domains overnight at 4°C. For competition studies, excess unlabeled ("cold") peptides were preincubated with GST or GST-NF- κ B p65 in protein-binding buffer for 4 h at 4°C, before addition of the radiolabeled peptides and overnight incubation at 4°C. The samples were then washed four times in binding buffer. The bound proteins were liberated by boiling in Laemmli sample buffer and were analyzed by SDS-PAGE and autoradiography.

Data Analyses

All numerical data are presented as mean \pm SEM and were analyzed by ANOVA. Significance was assigned at $P < 0.05$.

Results

Mapping the NF- κ B p65 Binding Domain on STAT3

We previously demonstrated that endogenous STAT3 α and NF- κ B p65 could be co-immunoprecipitated from nuclear extracts of activated mesangial cells and that *in vitro*-translated STAT3 α could be specifically retained by a GST-NF- κ B p65 fusion protein but not by GST alone (1). To define domains of STAT3 (Figure 1) that interact with NF- κ B p65, we synthesized 35 S-labeled proteins corresponding to each STAT3 α domain and tested their ability to bind specifically a GST-NF- κ B p65 fusion protein in the GST pulldown assay. The coiled-coil and DNA-binding domains were specifically retained by GST-NF- κ B p65 (Figure 2A), whereas the N-terminal domain, linker domain, SH2 domain, and transcriptional activation domain exhibited no interaction with NF- κ B p65 (Figure 2A), despite equivalent input amounts (not shown). These results suggest that the DNA-binding domain and coiled-coil domain contain essential motifs for their interaction with NF- κ B p65 and therefore could act as competitors for binding.

In competition assays, excess unlabeled coiled-coil domain or DNA-binding domain proteins were first incubated with GST-

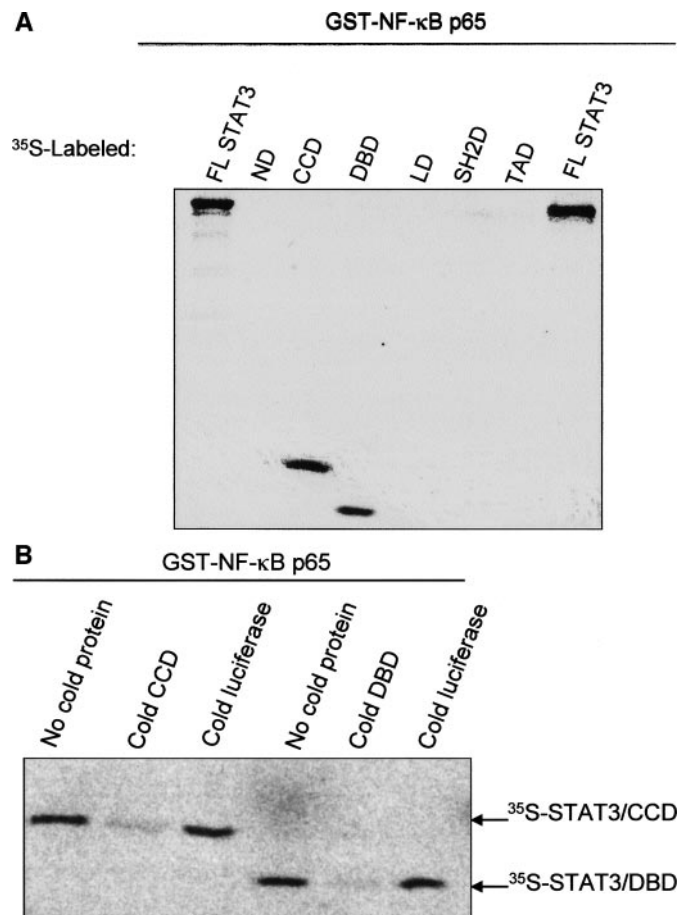


Figure 2. Mapping of the STAT3-interactive region in nuclear factor κ B (NF- κ B) p65 by glutathione S-transferase (GST) pulldown assays. (A) cDNA encoding full-length (FL) STAT3 α , the STAT3 N-terminal domain (ND), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (LD), SH2 domain (SH2D), and transcription activation domain (TAD) were *in vitro* transcribed and translated in the presence of [35 S]methionine. These translation products were incubated with GST-NF- κ B p65 and subjected to the GST pulldown assay as detailed in the Materials and Methods section. The products were analyzed by SDS-PAGE and autoradiography ($n = 4$). (B) The [35 S]STAT3/CCD and [35 S]STAT3/DBD translation products were incubated with GST-NF- κ B p65 in the presence or absence of a 25-fold molar excess of cold CCD, luciferase, or DBD proteins and then subjected to the GST pulldown assay as detailed in the Materials and Methods section ($n = 4$).

NF- κ B p65, and then [35 S]methionine-labeled coiled-coil domain, DNA-binding domain, or full-length STAT3 α proteins were added to the binding reaction. Incubation with the cold peptides dramatically decreased coiled-coil domain and DNA-binding domain binding to GST-NF- κ B p65 compared with unlabeled luciferase protein, which was used as a nonspecific protein-negative control (Figure 2B). These results indicate that the isolated coiled-coil domain and DNA-binding domain of STAT3 specifically interact with NF- κ B p65 *in vitro*.

Region L³⁵⁸ through I³⁶⁸ of the STAT3 DNA-binding Domain Is Critical for Its Binding to NF- κ B p65

For determining the minimal region of STAT3 required for NF- κ B p65 binding, a series of STAT3 deletion constructs

containing the linear sequence of the DNA-binding domain and linker domains (as in Figure 1: amino acids 342 to 585, 358 to 585, 369 to 585, 380 to 585, and 392 to 585) were generated and used in GST pulldown assays. The linker domain, which alone does not bind GST-NF- κ B p65 (Figure 2), was incorporated into the constructs to increase the molecular size so as to provide better size resolution on the SDS-PAGE gels. Equivalent amounts of 35 S-labeled DNA-binding domain + linker domain protein subdomains were again incubated with bead-bound GST-NF- κ B p65. In these experiments, omission of the region L³⁵⁸ through I³⁶⁸ of STAT3 resulted in greatly reduced binding, indicating that this region is necessary, sufficient, and the predominant region for GST-NF- κ B p65 binding (Figure 3).

Guided by these results, we next sought to identify specific residues within this region of STAT3 that might be important for STAT3-NF- κ B p65 interaction. Amino acid sequence alignment of six mammalian STAT isoforms revealed four conserved residues in the region L³⁵⁸ through I³⁶⁸ of STAT3: L³⁵⁸, N³⁵⁹, K³⁶³, and V³⁶³ (Figure 4A). Site-directed mutagenesis was performed to convert each of these four residues to alanine. A STAT3 cDNA encoding the DNA-binding domain and linker domain harboring all four of these mutations was expressed as a 35 S-labeled protein (termed “ Δ DBD” in Figure 4B) and tested for its ability to bind GST-NF- κ B p65. In contrast to the strong binding activity of the wild-type protein, this STAT3 mutant exhibited negligible binding to NF- κ B p65 (Figure 4B). These results confirmed that specific residues within the region L³⁵⁸ through I³⁶⁸ of the DNA-binding domain are necessary, sufficient, and predominant for the STAT3-NF- κ B p65 interaction.

Mutation of the region L³⁵⁸ through I³⁶⁸ of STAT3 relieves the STAT3-mediated transrepression of IL-1 β -activated endogenous NO production and the activity of iNOS promoter-reporter and κ B element-reporter genes. We previously demonstrated that overexpression of STAT3 inhibited IL-1 β -stimulated endogenous nitrite generation in mesangial cells (1). To prove the function of the STAT3 L³⁵⁸ to I³⁶⁸ region of the DNA-binding domain in the physical and functional interaction

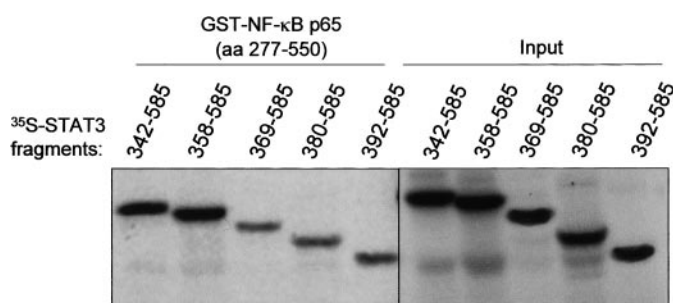


Figure 3. Deletion analysis mapping of region in the STAT3 DNA-binding domain critical for interaction with NF- κ B p65. A series of radiolabeled *in vitro* translation products corresponding to amino acids 342 to 585, 358 to 585, 369 to 585, 380 to 585, and 393 to 585 of STAT3 (see also Figure 1) were generated and subjected to the GST pulldown assay with GST NF- κ B p65 as described in the Materials and Methods section ($n = 4$).

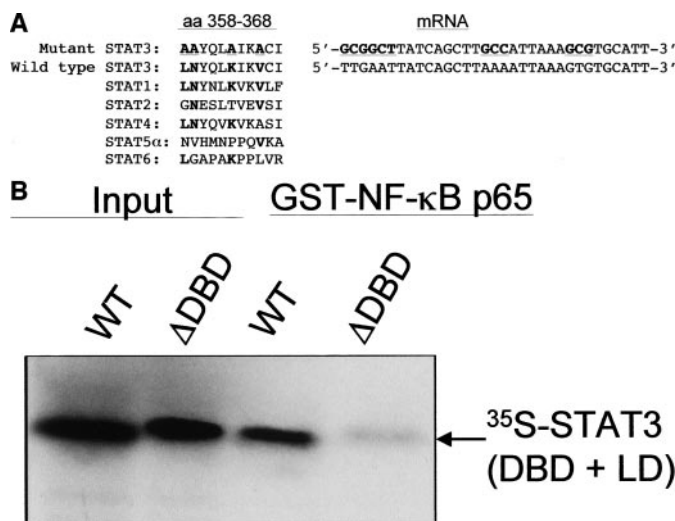


Figure 4. Site-directed mutagenesis in the DNA-binding domain of STAT3. (A) Sequence alignment of STAT proteins in the DNA-binding domain. Identically conserved residues are in bold. The four residues of STAT3 underlined and in bold were changed individually to alanine by site-directed mutagenesis. (B) Wild-type and a L³⁵⁸A, N³⁵⁹A, L³⁶⁰A, and V³⁶⁶A mutant of the [35 S]STAT3 DNA-binding domain and linker domain (amino acids 320 to 585) translation product (designated at “ Δ DBD”) were incubated with GST-NF- κ B p65 and subjected to the GST pulldown assay ($n = 4$).

with NF- κ B p65 in intact mesangial cells, we measured IL-1 β -stimulated nitrite production in cells transiently transfected with the wild-type STAT3 α expression plasmid pSG5-STAT3 α or plasmid pSG5-STAT3 α^{Δ DBD, which contains alanine mutations at L³⁵⁸, N³⁵⁹, K³⁶³, and V³⁶³ of the DNA-binding domain. As seen in Figure 5A, pSG5-STAT3 α but not pSG5-STAT3 α^{Δ DBD inhibited IL-1 β -stimulated nitrite generation. In fact, cells transfected with pSG5-STAT3 α^{Δ DBD exhibited higher nitrite levels than empty vector-transfected controls, suggesting a dominant-negative effect on endogenous STAT3 to limit its inhibitory action. As further proof of principle, transactivation/transrepression assays were performed in which mesangial cells were co-transfected with the iNOS promoter construct piNOS-luc together with pSG5-STAT3 α or pSG5-STAT3 α^{Δ DBD, and then IL-1 β -stimulated iNOS promoter activity was measured. In agreement with our earlier work, overexpression of wild-type STAT3 resulted in IL-1 β -stimulated iNOS promoter activity that was approximately 40% lower than that of the pSG5 vector-transfected controls (Figure 5B). In contrast, pSG5-STAT3 α^{Δ DBD did not significantly transrepress IL-1 β -stimulated iNOS promoter activity (Figure 5B). Finally, overexpression of wild-type STAT3 dramatically suppressed the IL-1 β -induced activity of an NF- κ B-dependent promoter that lacks STAT binding elements, in keeping with our previous results (1), whereas pSG5-STAT3 α^{Δ DBD did not (Figure 5C).

These results support the conclusion that the region L³⁵⁸ through I³⁶⁸ of STAT3 participates in the physical and functional interaction with NF- κ B to limit iNOS transcription in cytokine-activated mesangial cells. That STAT3-mediated in-

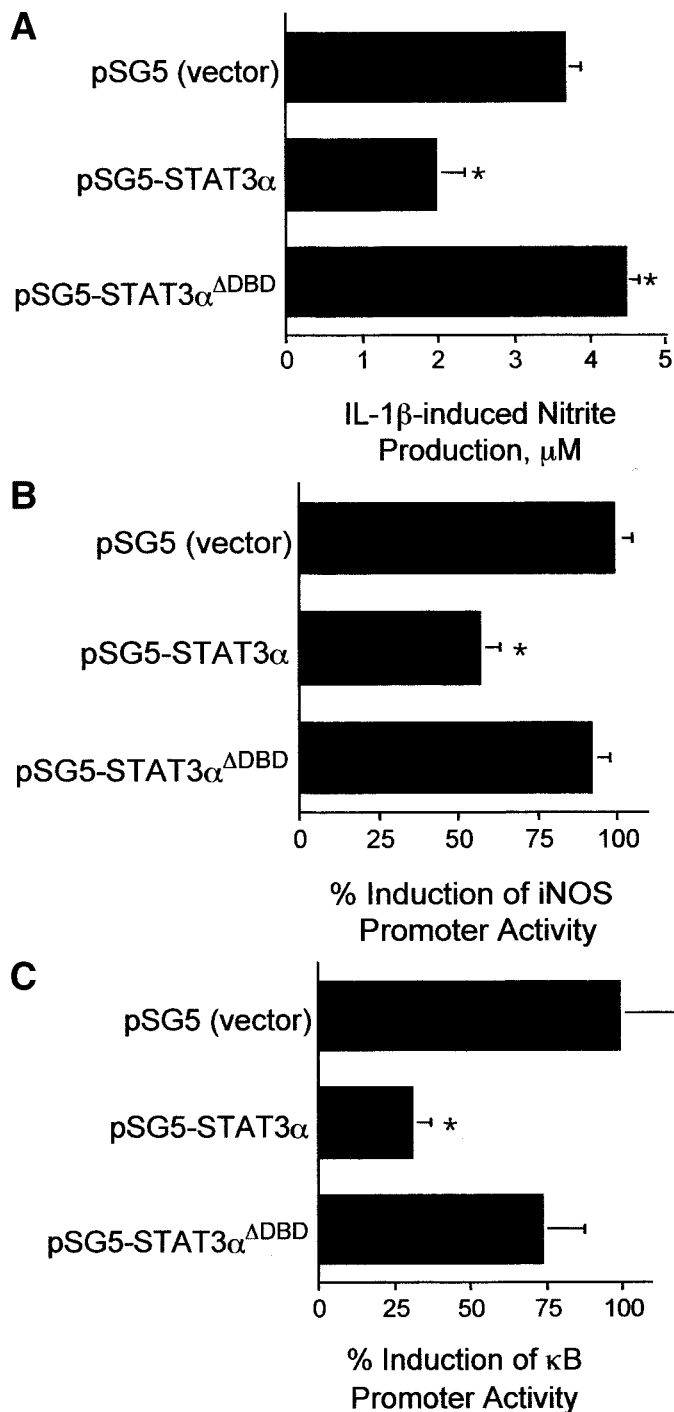


Figure 5. Mutation of the STAT3 DNA-binding domain relieves the STAT3-mediated inhibition of IL-1 β -induced nitric oxide (NO) production and activity of inducible NO synthase (iNOS) promoter-reporter and κ B element promoter-reporter genes. (A) Mesangial cells were transiently co-transfected with the wild-type STAT3 α expression plasmid pSG5-STAT3 α , pSG5-STAT3 α^{Δ DBD (which bears alanine substitution mutations at L³⁵⁸, N³⁵⁹, L³⁶⁰, and V³⁶⁶ in the DNA-binding domain), or the parent vector pSG5. Twenty-four hours after transfection, the cells were treated with vehicle or IL-1 β , and the nitrite content of the medium and protein content of the cells was measured ($n = 4$). * $P < 0.05$ versus pSG5 value. (B) Mesangial cells were transiently co-transfected with pSG5-STAT3 α , pSG5-STAT3 α^{Δ DBD, or the parent vector pSG5, together with piNOS-luc,

inhibition of NF- κ B did not completely suppress iNOS promoter activity likely reflects the probability that the inhibitory effect of the protein–protein interaction is not totally effective and that other cytokine-activated transcription factors are known to participate in iNOS promoter transactivation (23–25).

Discussion

Transcriptional activation of mammalian genes is now universally regarded as requiring the cooperative or antagonistic effects of many proteins. The present study builds on our earlier work in which we showed that one such interaction, between STAT3 and NF- κ B p65, functions to limit the ability of NF- κ B to transactivate the iNOS gene in glomerular mesangial cells without the need for STAT3 binding to cognate elements in the iNOS promoter and without limiting NF- κ B DNA-binding activity (1). Prolific synthesis of NO by iNOS can cause unintended injury to host cells during glomerulonephritis and other inflammatory diseases. Tight regulation therefore is required to terminate NO generation once the physiologic responses have been achieved. We now find that the coiled-coil domain and the DNA-binding domain of STAT3 interact with NF- κ B p65, especially a region of the STAT3 DNA-binding domain from residues L³⁵⁸ to I³⁶⁸. A peptide spanning this region of the DNA-binding domain efficiently and specifically competed for NF- κ B p65 binding in GST pulldown assays. Moreover, mutations of this region virtually abolished the STAT3:NF- κ B p65 interaction *in vitro* and relieved the effect of STAT3 to transrepress iNOS-mediated NO generation, iNOS promoter activity, and the induction of a κ B element-reporter gene in IL-1 β -stimulated mesangial cells. In the aggregate, our studies suggest a model in which STAT3, *via* direct interactions of its DNA-binding domain with NF- κ B p65, serves as a dominant-negative inhibitor of NF- κ B activity to suppress indirectly cytokine induction of the iNOS promoter in mesangial cells. This mechanism may serve to terminate NO production by activated iNOS after exposure to proinflammatory stimuli and to facilitate mesangial cell proliferation.

The STAT3 DNA-binding domain is fairly large compared with other such domains and presents potential points of interaction with proteins separate from the single surface that interacts with DNA. Thus, the opportunity of STAT to interact with additional molecules that may be bound to DNA, such as other transcription factors, certainly exists for the DNA binding domain of the STAT. Neither the L³⁵⁸ to I³⁶⁸ sequence nor

containing the wild-type iNOS promoter fused to the firefly luciferase gene, as well as with a *Renilla* luciferase expression plasmid. After transfection, the cells were treated with vehicle or IL-1 β , and the luciferase activity in cell lysates was measured ($n = 5$). * $P < 0.05$ versus pSG5 value. (C) Mesangial cells were transiently co-transfected with pSG5-STAT3 α , pSG5-STAT3 α^{Δ DBD, or the parent vector pSG5, together with p36B(-)(NF- κ B)₃-luc, containing a κ B element concatamer fused to the firefly luciferase gene, as well as with a *Renilla* luciferase expression plasmid. After transfection, the cells were treated with vehicle or IL-1 β , and the luciferase activity in cell lysates was measured ($n = 4$). * $P < 0.05$ versus pSG5.

its immediately neighboring residues contain any known motifs for protein–protein interactions. It remains possible, however, that tertiary structure rather than linear sequence presents such a motif. Because we previously demonstrated that STAT3 did not itself require binding to the iNOS promoter region for its ability to bind NF- κ B p65 and to inhibit NF- κ B–mediated activation of the iNOS gene (1), it is possible that STAT3 binding *via* the DNA-binding domain to its cognate DNA elements in the iNOS promoter and to NF- κ B are to a degree mutually exclusive in this cell and promoter context. Furthermore, because STAT3 overexpression does not limit NF- κ B DNA-binding activity (1), it is likely that protein–protein interactions between the two transcription factors alter the transactivation potential of NF- κ B either through conformational changes of the latter protein or through altered involvement of co-regulatory proteins.

The coiled-coil domain is commonly involved in protein–protein interactions, and it plays a pivotal role in the regulation of STAT3 tyrosine phosphorylation (17). *In vitro* GST pull-down assays identified a segment of STAT3 from residues approximately 130 to 358 that binds to the C-terminus of c-Jun (17). The coiled-coil domain of STAT3 interacts with the c-Jun SH2 domain to mediate receptor binding and induce tyrosine phosphorylation and tyrosine phosphorylation–dependent activities (17). The C-terminal domain of STAT3 negatively regulates receptor-binding activity only in the absence of the first α -helix of the coiled-coil domain (26). Another study demonstrated that a small SH2 domain–binding peptide blocked STAT3-mediated DNA binding activity, gene regulation, and cell transformation (27). In the aggregate, these data suggest that both protein–protein interactions and domain–domain interactions contribute to STAT3 function. In our study, however, although the coiled-coil domain interacted *in vitro* with GST-NF- κ B p65, it was the DNA-binding domain that exclusively mediated the negative regulation of cytokine induction of iNOS transcription and NO production, because mutations in this latter region completely relieved the transrepression caused by wild-type STAT3 (Figure 5). Accordingly, we have not yet pursued further analysis of the contributions of the coiled-coil domain to the functional and physical interactions of STAT3 and NF- κ B p65.

In addition to our example of STAT3 interacting with NF- κ B p65 (1), STAT5a was shown to inhibit NF- κ B signaling by competing for limiting amounts of coactivators necessary for NF- κ B–mediated gene transcription (12). However, the specific regions of interaction were not defined in that study. Recent studies have also shown that the activities of STAT can be modulated by the interactions with other DNA-binding proteins and non–DNA-binding proteins, such as coactivators. p300/CBP has been shown to interact with STAT1, STAT2, and STAT5 α as well as with NF- κ B to enhance target gene expression. A short region in the first α -helix of the coiled-coil domain and a portion of the DNA-binding domain, outside the region identified in the present report, of STAT3 were found to interact with another transcription factor, c-Jun, and cooperatively to activate transcription of the IL-6–inducible α 2-macroglobulin gene (28). In addition to NF- κ B, STAT

interact with a wide variety of factors, including p48/IRF9, these SMAD, Sp1, USF-1, c-Jun, PU.1, C/EBP, glucocorticoid receptor, NcoA-1, YY-1, TFII-1, and HMG-I(Y). In no other instance has the DNA-binding domain been implicated in protein–protein interactions. The coiled-coil domain mediates some of these interactions, but the STAT linker domain is also involved in transcriptional control. These latter domains do not seem to be significantly involved in STAT3 transrepression of the iNOS gene as shown here. Similarly, NF- κ B/Rel proteins have also been shown to interact with proteins involved in transcriptional regulation (29). Because the subunit composition of the NF- κ B/Rel complexes differs among different cell types and in response to different stimuli, it is possible that the function of activated STAT3 is regulated through the binding of different NF- κ B/Rel protein in NF- κ B p65-containing complexes, which bind to κ B sites in many genes differentially and function differently on diverse genes (30, 31). Moreover, cross-talk between NF- κ B/Rel proteins and other transcription factor families (32–35) might also modulate the function of Stat3–NF- κ B complexes in transcriptional activation. These additional levels of regulation remain to be analyzed.

STAT3 α governs, in a cell-specific way, the magnitude of iNOS induction in mesangial cells (1) and vascular smooth muscle cells (36). STAT3 plays an essential role in embryonic development, as well as in growth and differentiation of hematopoietic cells. Protein–protein interactions have been demonstrated to be important for the ability of STAT proteins to regulate target gene transcription. Therefore, understanding the mechanisms that control STAT3 activation and protein pairing may provide useful insights into these cellular processes. Moreover, the DNA-binding domain of STAT3 may be a useful target for drug design and screening of small molecules that inhibit STAT3 activity by disruption of binding to this domain.

Acknowledgments

This work was supported by National Institutes of Health Grants RO1-DK50745 and P50 GM-38529, a Department of Defense “DREAMS” grant, and endowment funds from The James T. and Nancy B. Willerson Chair (to B.C.K.). We thank Dr. David Tweardy (Baylor College of Medicine) and Dr. Bharat Aggarwal (The University of Texas M.D. Anderson Cancer Center) for their gifts of plasmids and Dr. Shuo Dong (Baylor College of Medicine) for helpful experimental suggestions.

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