Interleukin 1 Activates STAT3/Nuclear Factor-κB Cross-talk via a Unique TRAF6- and p65-dependent Mechanism*

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Interleukins (ILs) 1 and 6 are important cytokines that function via the activation, respectively, of the transcription factors NF-κB and STAT3. We have observed that a specific type of κB DNA sequence motif supports both NF-κB p65 homodimer binding and cooperativity with non-tyrosine-phosphorylated STAT3. This activity, in contrast to that mediated by κB DNA motifs that do not efficiently bind p65 homodimers, is shown to be uniquely dependent upon signal transduction through the carboxyl terminus of TRAF6. Furthermore, STAT3 and p65 are shown to physically interact, in vivo, and this interaction appears to inhibit the function of “classical” STAT3 GAS-like binding sites. The distinct p50 form of NF-κB is also shown to interact with STAT3. However, in contrast to p65, p50 cooperates with STAT3 bound to GAS sites. These data argue for a novel transcription factor cross-talk mechanism that may help resolve inconsistencies previously reported regarding the mechanism of IL-1 inhibition of IL-6 activity during the acute-phase response.

Interleukins (ILs) 1 and 6 play critical roles in inflammation and stress. One of the important homeostatic processes is the acute phase response (APR) in which various stress stimuli, such as microbiological products, injury, and neoplasia, result in the activation of endothelial cells, smooth muscle cells, and antigen-presenting cells such as macrophages, which in turn express cytokines such as IL-1, TNF, and IL-6. These cytokines can induce targets, such as endothelial, fibroblast, chondrocyte, osteoclast, and pituitary cells, to express factors important for both cellular and humoral immunity. These factors include IL-4, -5, -12, and -18 as well as secondary expression of additional IL-1, -6, and TNF. This organism-wide phenomenon has recently been referred to as the “danger response” (1).

The liver is an important component of the APR and has been exhaustively studied (2). Central to the activation of the liver is the induction of distinct gene collectives that are primarily under the control of three transcription factors, namely STAT3, NF-κB, and C/EBPβ. STAT3 is activated from a cytoplasmic non-tyrosine-phosphorylated monomer to a nuclear localized tyrosine-phosphorylated dimer by an IL-6-dependent signal transduction mechanism. Both C/EBPβ (also called NF-IL6) and NF-κB are activated either by IL-1 or TNF. C/EBPβ is activated by direct mitogen-activated protein kinase serine phosphorylation, which results in a conformational change of the constitutively inactive transcription factor (3, 4). NF-κB is activated via either serine or tyrosine phosphorylation of the various forms of the IκB inhibitor that is subsequently released and usually degraded by the proteasome (5). Although IL-6 can also activate C/EBPβ, it cannot activate NF-κB. The IL-6 responses mediated by STAT3 are known to be somewhat independent of those associated with C/EBPβ and especially NF-κB. In fact, evidence has accumulated arguing that many of the IL-6 responses are inhibited by agents that activate NF-κB (6–9). Yet, under some conditions the two responses are also cooperative. Although several models have been proposed to explain these apparently inconsistent observations, none is either entirely satisfactory or universally accepted (7, 9–11).

In the process of investigating such mechanisms, we discovered that an asymmetric functional relationship exists between STAT3 and NF-κB. This relationship is mechanistically dependent upon a protein-protein interaction between one form of NF-κB and monomeric apophosphorysine STAT3 (STAT3p50). We have observed that NF-κB p65 homodimers can cooperate with STAT3p50, when bound to a specific type of κB motif. Reciprocally, this interaction appears to inhibit function of “classical” STAT3 GAS-like binding sites. In contrast, NF-κB p50 can cooperate with STAT3 bound to GAS sites. The specificity of the response is not only associated with the specific DNA binding site, but also the signal transduction pathway, which appears to rely upon the carboxyl-terminal meprin and TRAF homology (MATH) domain of TRAF6 for the selective activation of p65 homodimers. Together these data argue for a novel transcription factor cross-talk mechanism that may explain many of the controversial observations associated with APR and the activation of distinct forms of NF-κB.
EXPERIMENTAL PROCEDURES

Cell Lines—Hep3B cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mm t-glutamine, and 0.5% penicillin-streptomycin in humidified 10% CO2 and 90% air at 37 °C. HEK293 cells were derived from HEK293s (ATCC) and then stably transfected with pcDNA3.1 expression vector (Invitrogen) containing the cDNA sequence for the human IL-1 type I receptor chain (IL1R1) introduced by recombinant PCR subcloning. These cells expressed a qualitatively similar response to the parental HEK293. However, the presence of IL1R1 amplified the response, generating a higher sensitivity. Selection for stable transfection was accomplished in the presence of 400 μg/ml G418. The resulting cells expressed high levels of IL-1 type I receptor and are referred to in the text as HEK293/ILR1 cells.

Oligonucleotides and Antibodies—For all of the oligonucleotide sequences shown, the consensus factor binding sites are underlined. High affinity siss-inducible element (hSIE), which binds both STAT3 and STAT1 (12), 5′-ACCTGTCGATGATTCCCTAAATCCTTGTCGTGCA-3′; STAT3-specific 4-spaced-like sequence (13), 5′-ACCTCTATTCCTGGAAAAGCCTCATG-3′; human IL-8 promoter (14) (−88 to −65), 5′-GAATTCCTGGAATACTCCGTGCA-3′; human ICAM-1 promoter (15) (−194 to −171), 5′-TTATGCTCTGGAAATTCGAGGCTG-3′; human Bcl-x gene promoter (16) (−238 to −215), 5′-GGGGGGGCGGAGGCCTCCAGGGAGTG-3′; human iNOS gene promoter (17) (−5790 to −5813), 5′-CTGGTTCTGCTGGGACCGCTT-3′; and the wild-type and mutant GAS/oligonucleotides, as sequences described in Fig. 4, were synthesized by Operon (Valencia, CA).

The GAS/sb (9-bp NF-κB) 5′-GATCTCTGCGAGATCTCCATGATC-3′ used in Fig. 1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Antibodies obtained from Santa Cruz Biotechnology were as follows: anti-NH2-terminal STAT3s, anti-internal STAT3s, anti-COOH-terminal STAT3s, anti-p56, anti-p58, anti-STAT1, and anti-STAT3 phosphotyrosine 705.

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs were performed essentially as described previously (18, 19). Briefly, 10 μg of whole cell extracts or nuclear extracts was preincubated for 20 min at room temperature in 15 μl of buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 4%glycerol, 40 μM NaCl) containing 1 μg of poly(dI-dC), and each oligonucleotide was labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and γ-32PATP (6,000 Ci/mmol; PerkinElmer Life Sciences). Protein-DNA complexes were resolved on 4% TBE polyacrylamide gels and analyzed with or without specific antibody or unlabeled hSIE oligonucleotide.

Constructs—Luciferase reporter plasmids were constructed containing the sequences used as EMSA probes (see above) inserted upstream (between unique KpnI and NheI sites) of the minimal (−56 to +109) murine c-fos promoter (20), which had previously been inserted into the pGL3 Basic promoter (Promega, Madison, WI). The GAS/sb reporter used for most of the studies harbored four copies of the 24-bp oligonucleotide containing the 9-bp sb sequence described above, under “Oligonucleotides and Antibodies.” These four sequences are separated by spacers of 12, 32, and 12 bp in order from the upstream-toward the downstream-most element. An additional 59 bp separates the last element from the −56 position of the c-fos promoter. The hSIE GAS reporter contained two copies of the oligonucleotide described above, spaced 15 bp apart and separated from the c-fos promoter by 47 bp. The 4-spaced-like GAS reporter contained a 460-bp region of the upstream 9-bp sequence described above containing oligonucleotide located 94 bp upstream of the c-fos promoter. The wild-type and mutated GAS/sb reporters described in Fig. 4 each contained two copies of the same sequence located 47 bp upstream of the c-fos promoter. The MHCβ reporter (MHCβ-luc) was from Dr. T. Born (21) and contained four tandem copies of the described oligonucleotide located 3 bp upstream of the c-fos promoter. IL-8 reporter (IL-8-luc) was provided by Dr. T. A. Libermann, which contains the IL-8 promoter. IL-6 reporter (IL-6-luc) was from Dr. P. Oettgen. All plasmids used for transfections were prepared by the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA).

TRANSFECTIONS AND LUCIFERASE ASSAYS—Cells were transfected with the transfection reagent FuGENE6 (Roche Applied Science) at 3 μl of reagent/μg of DNA according to the manufacturer’s instructions. A total of 0.15–0.5 μg of DNA, including the luciferase reporter construct, expression vectors, and 0.01 μg of pRL-TK-RLuc (Promega), was added to 1.44 mg/ml transfection efficiency was monitored by use of the internal control.

Immunoblots—For preparation of whole cell lysis, cells were lysed in radiommune precipitation assay buffer with 10 mg/ml phenylmethylsulfonyl fluoride, aprotinin, proteinase inhibitors (CompleteTM, EDTA-free; Roche Applied Science). Nuclear extracts were prepared using a kit (Sigma) according to the manufacturer’s instructions. The protein amount for each sample was measured using a Bradford assay (BioRad) and adjusted to be equal in all samples. Immunoprecipitation and Western blot analysis with enhanced chemiluminescent detection (SuperSignal WestFemto System, Pierce) were carried out by standard methods (24). SDS-PAGE gel was run with 40% acrylamide using a 29:1 ratio of acrylamide to bisacrylamide. Molecular weight standards (BioRad) were used for determining molecular mass.

Chromatin Immunoprecipitation (ChIP)—Hep3B cells were grown in 100 × 20-mm plates to 75% confluence and were treated with 10 ng/ml IL-1β and 50 ng/ml IL-6. After 15 min, ChIP was performed using a kit from Upstate USA. In brief, proteins of both treated and untreated cells were immunoisolated linked to DNA containing 33% formaldehyde to a final concentration of 1% for 15 min. Cells were washed with ice-cold phosphate-buffered saline containing protease inhibitor mixture (Sigma), scraped, and washed three times. Cells were lysed with 300 μl of SDS-lysis buffer containing protease inhibitor, and DNA was sonicated (10 W on a Fisher model 100 Sonic Disembratur for 5 s at 0–20°) to generate 200–1,000-bp length, and samples were centrifuged at 12,000 rpm × 8 min. Sonicated cell supernatant was diluted 10-fold with ChIP dilution buffer (Upstate USA).

Immunoclearing was accomplished by incubating with sheared salmon sperm DNA and protein A/G-Sepharose. Samples were rotated for 4°C for 30 min and incubated with specific antibodies overnight. Sepharose beads were harvested by centrifugation (3,000 rpm × 10 min) and washed with ChIP wash buffer (Upstate). Beads were incubated with proteinase K for 45 min. DNA was eluted from the beads with elution buffer (1% SDS, 0.1 M NaHCO3), and then DNA cross-links were reversed by incubating the sample 4 h to overnight at 65°C in 0.2 M NaCl.

Final purification of DNA used a PCR minielute kit (Qiagen) followed by PCR amplification using specific primers and Fast Start Taq polymerase (Roche Applied Science). The PCR amplification conditions were 4 min at 94 °C followed by 27 cycles of 30 s at 94 °C, 30 s at 54 °C, and 45 s at 72 °C. Synthesis was terminated by incubation for 15 min at 72 °C. The human IL-8 gene primers were 5′-TGCATCAATAAACAGTGAGGG-3′ and 5′-ACCTGCCCTGATTTCTGACA-3′ separated from the c-fos promoter by 13 bp. Synthesis was terminated by incubation for 15 min at 72 °C. The human IL-8 gene primers were 5′-TGCATCAATAAACAGTGAGGG-3′ and 5′-ACCTGCCCTGATTTCTGACA-3′ separated from the c-fos promoter by 13 bp.

RESULTS

IL-1 Induces a STAT3p65 Complex to Bind DNA—It is known that IL-1β induces the transcription of many genes via members of the REL/NFκB family of transcription factors. The REL factors, such as p50, p52, p65, c-Rel, RelA, and RelB, function as dimers and have been shown to be both differentially activated (25–27) and to possess distinct target DNA binding site specificities (28, 29) that depend upon dimer composition. The p50 and p52 proteins do not possess a transactivation domain (TAD) and can either inhibit as homodimers or potentiate the activity of other REL factors upon heterodimerization. Fig. 1 demonstrates that Hep3B cells treated for 15
nucleotide known to be a consensus sequence for STAT3 homo- and heterodimers (Fig. 1B, lane 9). As expected, EMSA analysis using the hSIE GAS probe revealed that, in contrast to IL-6, IL-1 could not induce STAT3 binding (data not shown). Interestingly, complex II could only be detected to bind MHCxB probe after very long exposure to film (not shown), arguing that the NF-κB site in the MHCxB probe binds only p50/p65 heterodimers strongly.

It is unclear why there is an absence of an effect for N-STAT3 antibody (Fig. 1B, lane 5). All the antibodies were tested independently on EMSA using extracts from IL-6-treated Hep3B cells and a labeled hSIE probe (not shown). The EMSA revealed the expected three complexes corresponding to STAT3 and STAT1 homo- and heterodimers. All the antibodies reacted with the appropriate complexes, except for N-STAT3, which reacted with the STAT3 homodimer complex, but not with that corresponding to the STAT1/STAT3 heterodimer. It is not known whether this is because the antibody recognizes the likely complex that occurs between STAT amino termini or whether there is a distinct conformational change for the heterodimer.

Because complex II appeared to be completely dependent upon both p65 and STAT3, we looked for a protein-protein interaction between these two proteins. Following IL-1 stimulation, cell lysates were prepared and immunoprecipitated with either anti-p65 or anti-STAT3 antibodies. Fig. 1D shows that STAT3 is coimmunoprecipitated with p65, suggesting that p65 interacts with STAT3 in vivo. The above results show that p65 can make a complex with STAT3 both in vitro, in the presence, and in vitro, in the absence of DNA. This complex binds to an overlapping GAS/xB motif, but not to the MHCxB sequence, which does not reveal any obvious GAS-like sites.

STAT3 and p65 Cooperate Functionally on a Specific DNA Binding Site—To characterize functionally the in vitro DNA binding, we prepared luciferase reporter constructs containing multiple copies of various binding sites positioned immediately upstream of the murine c-fos core promoter. These included reporters containing the GAS/xB and MHCxB sequences, as well as consensus GAS sequences such as the hSIE and 4spC, which are devoid of any obvious xB-like motifs. These constructs were transfected into Hep3B cells, and luciferase activity was assayed in response to stimulation with either IL-1 or IL-6 (Fig. 2A). Treatment of Hep3B cells with IL-1 resulted in strong activity for the GAS/xB and MHCxB-luc reporters, but not for the two GAS-luc reporters. This activity appeared to be dependent upon NF-κB, as judged by its sensitivity to inhibition by overexpression of a mutated form of IκBα, which serves as a strong NF-κB negative (dn-IκB in Fig. 2A). In contrast, IL-6 strongly activated the two GAS-luc reporters, but only weakly activated GAS/xB-luc. As expected, the MHCxB-luc reporter did not respond to IL-6.

Fig. 1B shows that STAT3 was induced by IL-1 to bind to the GAS/xB sequence as an apparent complex with NF-κB p65. Therefore, we investigated whether the expression of STAT3 could induce the GAS/xB-luc reporter. Fig. 2B shows that STAT3 alone, as expected, could not induce GAS/xB-luc. This is in contrast to what was observed for cotransfection with an expression vector coding for NF-κB p65. This is probably the result of an expression level that exceeds that of the endogenous IκBα inhibitors. Coexpression of both STAT3 and p65 resulted in a cooperative response. Interestingly, this cooperativity appeared to be independent of IL-6 stimulation as well as the ability of STAT3 to be tyrosine-phosphorylated, as judged by the activity of a nonphosphorylatable Y705F mutation in STAT3 (mSTAT3). To confirm that the product of the Y705F mutated STAT3 expression vector was not tyrosine-phospho-
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Fig. 2. STAT3 and p65 functionally cooperate on a specific binding site. Various luciferase reporter constructs (GAS/κB, MHCκB, and hSIE-GAS) were transfected into Hep3B cells, which were subsequently stimulated with either IL-1 or IL-6 prior to the measurement of luciferase activity. A, plasmid coding for the expression of an unphosphorylatable mutated IκBα was used for inhibition of NF-κB activity. B, expression vectors coding either for wild-type (Stat3) or mutated, unphosphorylatable, STAT3 (mStat3) were cotransfected along with a p65 expression vector into cells that were either treated or not with IL-6. C, mutated STAT3 (mStat3) expression vector was transfected and stimulated or not with either IL-1 or IL-6. D, IL-1 treatment of cells was studied for possible effects on STAT3 activity using a GAS-luciferase reporter. Cells were treated with IL-1, IL-6, or both for 15 min. Also, IL-1 pretreatment (pre) for 15 min before the addition of IL-6 was examined. Western blotting employed either anti-COOH-terminal STAT3 a (Stat3) or an anti-STAT3 phosphotyrosine 705 (P-Stat3).

rylated, the vector was cotransfected along with the GAS-luc reporter. Fig. 2C shows that the Y705F mutation resulted in dominant negative activity for the GAS-luc reporter, as reported previously (32, 33). It is well known that the IL-6-induced tyrosine phosphorylation of STAT3 is necessary for dimerization and DNA binding. The observation that STAT3aPTyr can cooperate with p65 suggests that tyrosine phosphorylation is not necessary for formation of the STAT3-p65 complex. The cooperative behavior between STAT3 and p65 was only observed for GAS/κB-luc, not MHCκB-luc. The results shown in Fig. 2D argue that IL-1 could neither efficiently tyrosine phosphorylate STAT3, nor interfere with tyrosine phosphorylation by IL-6. This argues that IL-1 inhibition of GAS site activation during APR is not likely via induction of SOCS3 (34), a Janus kinase inhibitor that mediates LPS- and TNF-dependent inhibition of STAT3. The observation that pretreatment of Hep3B cells with IL-1 inhibits IL-6 induction of a GAS-luc reporter, while not having an effect on STAT3 tyrosine phosphorylation, argues that SOCS3 is probably not involved in the observed activity inhibition.

p65 Inhibits, Whereas p50 Activates, a GAS-dependent Promoter—The observation that IL-1 induces the formation of a functionally cooperative interaction between p65 and STAT3aPTyr, on a composite GAS/κB-luc reporter, while inhibiting the activation of a GAS-luc reporter, prompted investigation of how different forms of NF-κB might affect the induction of a GAS-dependent reporter. Fig. 3 shows that cotransfection of expression vectors for either the p65 or p50 form of NF-κB resulted in distinct responses for a GAS-luc reporter. As expected, only IL-6 (and not IL-1, p50, and p65) induced transcription from the GAS-luc reporter. Expression of p65 inhibited the IL-6-dependent activity, whereas p50 cooperated with IL-6. As a positive control, expression of STAT3 in the presence of IL-6 revealed strong activity. These data argue that the activation of p65 by IL-1 may be responsible for both the inhibition of GAS-dependent genes as well as the observed cooperativity for the GAS/κB-luc reporter. Furthermore, it appears that TAD-deficient p50, in contrast to p65, can cooperate with IL-6 to activate a STAT3-dependent reporter containing APR class 2 GAS sites.

Cooperativity between STAT3 and p65 Depends upon the Binding of p65 to DNA—The observation that the composite GAS/κB sequence, in contrast to that of IgκB, both binds and functionally cooperates with STAT3, taken together with the observation that STAT3 and p65 can interact in the absence of DNA (Fig. 1D), suggests that either the presence of the GAS motif or differences between the nature of the two different κB sequences may be responsible for the observed specificities. In an attempt to evaluate the importance of the intact GAS site in the overlapping GAS/κB sequence, mutations were introduced into the nonoverlapping portion of each half-site (Fig. 4A). Fig. 4B shows that mutation of the κB (mNfκB), but not the GAS (mGAS) half-site eliminates formation of both DNA-binding complexes. In contrast, mutation of the GAS half-site has no effect on DNA binding.

To confirm this, the same sequences used as EMSA probe were incorporated into luciferase reporters and assayed for
activity. Fig. 4C shows that IL-1 activates both the wild-type and mGAS-luc but not mNFκB-luc, which is consistent with the EMSA data. Furthermore, as in Fig. 2B, p65 and STAT3 Y705F cooperate on both the composite GAS/xB and mGAS-luc reporters (Fig. 4D). Taken together, the STAT3-p65 complex likely binds to DNA primarily through the 9-bp p65 homodimer xB motif and not the GAS site. This suggests that the presence of the GAS site may not be critical for the cooperativity between the two factors.

**Other Proinflammatory Genes Contain Similar p65 Sites That Recruit STAT3**—It is known that numerous proinflammatory genes have 10–11-bp functional xB binding sites, of the form GGNNG−5CCC, in their promoters. Therefore, we examined a small collection of genes reported to be dependent upon NF-κB activation (Fig. 5A). Two genes (Bcl-x and iNOS) fit the 10–11-bp consensus, whereas two others (IL-8 and ICAM-1) have the shorter 9-bp xB site, similar to the STAT3-p65 complex binding GAS/xB site. However, in contrast to GAS/xB, neither of these contains an obvious GAS-like motif predicted to bind STAT3. Using these sequences as EMSA probes revealed that IL-1-induced a STAT3-p65 complex binding to IL-8 and ICAM-I, but not to the Bcl-x and iNOS sequences (Fig. 5B). Because the IL-8 promoter was reported previously to be dependent upon p65, but not p50 (29), a 1,521-bp IL-8 promoter fragment containing the 9-bp p65 homodimer-like site was tested. The IL-8 reporter was activated either by IL-1 or p65, but not by IL-6 (Fig. 5, C and D). Furthermore, the IL-8 promoter, in the absence of any GAS-like motif, revealed the same sort of p65 and STAT3 cooperativity observed for the 9-bp xB site derived from the GAS/xB sequence (Fig. 5D).

Further demonstration of in vivo p65 complex formation with STAT3 utilized ChIP to visualize direct recruitment of these factors to chromatin in response to appropriate signals. Fig. 5E, upper panel, reveals that, as expected, IL-1 and IL-6 rapidly activate, respectively, p65 and STAT3 to bind to IL-8 promoter chromatin (lanes 5 and 9). Also, as expected IL-6 does not activate p65 binding. However, IL-1 does activate STAT3 binding (lane 8). Furthermore, the IL-1-activated STAT3, in contrast to STAT3 activated by IL-6, is not tyrosine-phosphorylated. Therefore, it appears as though the activation of STAT3 binding to chromatin by IL-1 occurs via a nonconventional pathway that does not require tyrosine phosphorylation. This is consistent with the observation that unphosphorylatable STAT3 activating IL-8 occurs via a nonconventional pathway that does not require tyrosine phosphorylation. This is consistent with the observation that unphosphorylatable STAT3 of cooperative activation of transiently transfected p65-dependent reporter sequences (see Figs. 2, 4, and 5C).

The binding of phosphotyrosine STAT3 is not likely to be to the 9-bp xB site, but rather to some GAS-like sequence. The region of the IL-8 gene promoter, between −359 and +10, which is amplified by the PCR primers, contains several consensus GAS sites and one STAT3-like site at −243/−250. It should be noted that although the results demonstrate a clear interaction of tyrosine-phosphorylated STAT3 with the IL-8 gene promoter in response to IL-6 treatment, IL-6 alone cannot induce the promoter (Fig. 5C). Therefore, the binding of the phosphotyrosine STAT3 dimer may either be insufficient or unimportant for the activation of this gene.

**Activation of p65 by the TRAF6 COOH-terminal MATH Domain**—The DNA binding and activation patterns for promoters containing either the 10–11-bp MHCxB-like REL heterodimer or the shorter 9-bp p65 homodimer binding sites argues for a model in which the key component responsible for the functional distinction between the two types of sequences is the effective binding of p65 homodimers to DNA. This is supported by the functionally cooperative interaction of p65 with nonactivated STAT3Y705F, in the likely absence of direct STAT3 bind-
ing to DNA. Consistent with these data is a model in which p65/65 and not p50/p65 is capable of generating a complex that acts cooperatively to activate transcription via direct p65/p65 DNA binding, while inhibiting the transactivation potential of STAT3 for promoters containing a cognate (i.e. GAS-like) binding site.

The IL-1/LPS receptor signal transduction pathway is distinguished from all other receptor-mediated REL/NF-kB activation pathways because of its exclusive use of only TRAF6 as an intermediary signaling molecule. In contrast, other receptors such as the various TNF family receptors either do not use TRAF6 or employ TRAF6 along with other TRAFs. This is significant because TRAF6 is the only TRAF protein that can directly bind to and activate c-Src tyrosine kinase (35). The function of TRAF proteins as signal transducers also appears to be dependent upon the ability of these molecules to target the cell membrane, which may be dependent upon the interaction with membrane-bound Src family kinases (36). Supporting this, the attachment of a membrane-targeting Src-like myristoylation signal to an inactive form of TRAF has been shown to support activity (23).

Therefore, we used ligand-independent dominant positive induction of 293/IL1RI cells with various TRAF6-coding expression vectors cotransfected with either the 9-bp GAS/H9260 B- or the 10–11-bp MHC/H9260 B-luc reporter vectors. The TRAF6 expression vectors represented various truncated forms (Fig. 6A) that were ligated into vectors that either did or did not add a 16-additional amino acid (MGSSKSKPKDPSQRRR) v-Src myristoylation target to the amino terminus of the expressed protein. As shown in the top two panels of Fig. 6B, transfection of an expression vector coding for full-length unmodified TRAF6 resulted in dominant positive, ligand-independent, activation of both GAS/H9260 B and MHC/H9260 B-luc. Consistent with previous reports, only full-length TRAF was active (37). In contrast, TRAF6 constructs possessing the amino-terminal myristoylation signal (Fig. 6B, bottom panels) revealed a striking increase.

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Fig. 6. Activation of p65 homodimers may be mediated by a mechanism that requires the COOH-terminal MATH domain. A, simplified TRAF6 schematic showing the relative locations of the N-terminal finger domain and the COOH-terminal MATH domain. Numbers locate amino acid positions. B, HEK293R cells were transiently transfected with either wild-type or various deletion mutants of a TRAF6 expression vector, which was used to demonstrate specific genomic chromatin engagement. Upper panel, antibodies specific for NF-kBp65 (p65) and unphosphorylated STAT3 (C-ter). Lower panel, specific tyrosine-phosphorylated STAT3 (P-Y705) antibody was used to compare the activation state of the protein when bound to chromatin following cell treatment with either IL-1 or IL-6.

Fig. 5. Some inflammatory genes contain similar STAT3/p65 binding sites. A, the sequences of gene-specific oligonucleotide probes used for DNA binding studies are shown with + sites boxed. IL-8 and ICAM-I gene sequences are represented as 9-bp sites. Bcl-x and iNOS are represented as 10–11-bp sites. B, EMSA was performed using whole cell extracts from IL-1-stimulated Hep3B cells. The supershift employed anti-p50, p65, and anti-COOH-terminal STAT3 antibodies (Ab). C, cells were transfected with an IL-8 reporter plasmid as indicated with or without IL-1 and IL-6 treatment for 3 h. D, cells were transfected with expression vectors for p65, STAT3, mutant STAT3 (mStat3) as indicated, and IL-8-luc activity was measured. E, IL-8 gene ChiP analysis for IL-1- and IL-6-treated cells was used to demonstrate specific genomic chromatin engagement. Upper panel, antibodies specific for NF-kBp65 (p65) and unphosphorylated STAT3 (C-ter). Lower panel, specific tyrosine-phosphorylated STAT3 (P-Y705) antibody was used to compare the activation state of the protein when bound to chromatin following cell treatment with either IL-1 or IL-6.
only in GAS/κB activity exclusively for subdomain constructs containing the COOH-terminal MATH domain. This GAS/κB-specific activity was demonstrated to be absolutely dependent upon NF-κB because cotransfection with dominant negative IκB (IκBα) inhibited all activity. These data support an argument that the MATH domain may be important for downstream activation of the 9-bp p65/p65-dependent reporter, but not for the reporter containing the 10–11-bp p50/p65 site.

**DISCUSSION**

Cross-talk between transcription factors has become a commonly recognized mode of gene regulation. Such cross-talk can both inhibit and activate transcription. For example, in the case of NF-κB, a physical interaction with several different C/EBP bZIP factors results in the activation of C/EBP-dependent transcription and the inhibition of transcription dependent upon NF-κB (38). Functional and physical interaction between NF-κB and other bZIP factors, such as Fos and Jun, has also been reported (39). NF-κB cooperates with STAT6 (40–42) and can be either cooperative or inhibitory with STAT1, depending upon cell type (43). STAT3 has also been reported to bind Jun (44, 45) and C/EBPβ (46).

In this report we have demonstrated that cross-talk between NF-κB p65 and STAT3 contributes to cytokine-mediated activation of a DNA binding site that has a higher relative affinity for p65 homodimers than does a classical κB motif. We also demonstrate evidence for a novel mechanism of IL-1-mediated APR inhibition that is independent of the involvement of the SOCS3 Janus kinase inhibitor, which has been proposed to be the primary NF-κB-dependent inhibitor of STAT3 (47). This SOCS3-independent inhibitory mechanism involves an interaction between p65 homodimers and STAT3 which results in the inability of STAT3 to bind to and activate GAS site-dependent genes. In contrast, NF-κB p50 appears to cooperate with STAT3. This results in transactivation of APR class 2 GAS site-dependent genes. This is similar to what has been reported previously for p50 with STAT6 on the mouse germ line immunoglobulin Ce gene (42). Fig. 7 presents a schematic model for this mechanism.

The cooperativity that we report here between STAT3 and NF-κB p65 does not require STAT tyrosine phosphorylation. This is in contrast to the absolute requirement for such phosphorylation when STAT3 binds to GAS sequences (32, 33, 45). This is based on the observation that a non-tyrosine-phosphorylatable STAT3 (Y705F) can induce the effect, which is also not dramatically increased by treatment of cells with IL-6. Additionally, we have demonstrated that following IL-1 treatment of cells, both NF-κB p65 and non-tyrosine-phosphorylated STAT3 are recruited in vivo to chromatin in the region of the IL-8 gene promoter. This is in contrast with the situation following IL-6 treatment in which tyrosine-phosphorylated STAT3 is recruited and p65 is not (Fig. 5E). Although non-tyrosine-phosphorylated STAT3 has not been reported previously to be functional in activating transcription, this phenomenon has been reported for STAT1 (48, 49). Interestingly, although IL-1 has been reported to induce tyrosine phosphorylation of STAT3 (50–52), our results have shown that the amount of IL-1-induced tyrosine phosphorylation in Hep3B cells is minuscule compared with that induced by IL-6 (Fig. 2D) and can only be seen on Western blots upon long exposure or for other cell types (52). The p50 and p65 forms of NF-κB are known to possess distinct in vitro DNA binding specificities (53) that can reflect differential gene targeting (26, 29). For example, our results argue that the p65 homodimer, and not a p50/p65 heterodimer, interacts with STAT3ΔPTyr, because p65 homodimers do not bind efficiently to the NF-κB p50/p65 (10 or 11 bp) κB site (Fig. 1C).

**Fig. 7.** Schematic representation of the proposed mechanism for STAT3-NF-κB p65 cross-talk. Activation of NF-κB p65 homodimers uniquely results in recruitment of STAT3, resulting in preferential binding to 9-bp-long κB sites (such as the GAS/κB, IL-8, and ICAM-1 elements) that possess a higher relative avidity for p65 homodimers than for p65 heterodimers. This results in an increase in transcriptional activity for this type of site, presumably through the physical interaction with STAT3. The sequestration of STAT3 by p65 is also proposed to reduce the amount of STAT3 available for phosphorytrosine activation by IL-6, resulting in inhibition of IL-6-responsive APR class II STAT3 GAS sites. In contrast, p50 and STAT3 appear to cooperate on APR class 2 GAS sites. Although full-length TRAF6 can activate p65 homodimers as well as p50 heterodimers, overexpression of the TRAF6 MATH domain with the myristoylation signal specifically appears to activate only reporters containing the 9-bp-long p65 homodimer binding site. The + indicates a pathway (dashed arrow) activated by full-length/wild-type TRAF6, but not by the TRAF6 MATH domain containing the myristoylation signal.

Beyond the protein-protein interaction, the molecular nature of the mechanism involved in the cooperativity between STAT3 and NF-κB p65 is unclear. The STAT3 TAD may be tethered to NF-κB, providing additional transactivation potential. Alternatively, interaction of STAT3 with NF-κB may potentiate activity either by altering NF-κB conformation or interfering with IκB binding to, and inhibition of, NF-κB. In preliminary studies, we have observed that expression of a STAT3 protein mutated at a serine residue (S727A) targeted by p38 mitogen-activated protein kinase that is required for maximum TAD function on GAS sites (32), not only fails to enhance IL-1 induction of the GAS/κB reporter, but inhibits activity (data not shown). This result suggests that the STAT3 TAD may not be the site of protein-protein interaction because the S727A mutated protein appears to be acting as a dominant negative for IL-1 action on GAS/κB, which would require interaction with p65. The observation that both STAT3 and STAT6 (42) can cooperate with TAD-less p50 on classical GAS sites suggests that the REL homology domain, and not the TAD, may be the site of protein-protein interaction with p65. Therefore, the STAT3 TAD may be playing a direct role in supporting trans-
activation. This may occur as a result of STAT3 protein tethering to the p65 DNA binding REL domain in the absence of a cognate STAT DNA binding site. This is similar to what we and others have demonstrated for other transcription factors (54, 55).

Hepatocytes express a collection of APR genes in response to microbial stimuli, injury, and neoplasia. These genes are categorized as belonging to one of two general classes (2). Class 1 genes (e.g. α2-acid glycoprotein, C-reactive protein, and SOCS3) are induced by IL-1, LPS, and TNF, and NF-κB and p38 mitogen-activated class 2 sites via the binding of NF-κB. GAS-dependent activation is dependent upon proteasome (56) and p38 mitogen-activated class 2 genes is not absolute (9). Other genes are inhibited by class 1 gene induction, whereas the class 1 genes are either independent of or cooperative with class 2 activation. A variety of mechanisms have been forwarded to explain this complex regulation. These include inhibition of GAS-dependent class 2 sites via the binding of NF-κB to overlapping sites (7, 8) and by the induction of SOCS3, which inhibits STAT3 activation by the IL-6 receptor (10, 11, 47). The mechanism for the cooperativity between class 2 activation and class 1 genes is unclear but could be related to the ability of class 1-inducing agents. Although our observations suggest that inhibition of GAS sites is actually accomplished by competitive binding of NF-κB to overlapping GAS motifs, it has been reported that the inhibition is dependent upon proteosome (56) and p38 mitogen-activated protein kinase function (10, 11). This is consistent with a role both for NF-κB activation and serine phosphorylation of the STAT3 TAD. Our results are consistent with the involvement of NF-κB in inhibiting class 2 gene expression. However, rather than being dependent only upon competitive binding to overlapping κB sites or to the inhibition of STAT3 activation by SOCS3, the formation of a protein-protein interaction between p65 homodimers and STAT3 may also be important.

We also show that overexpression of a COOH-terminal TRAF6 MATH domain possessing an amino-terminal myristoylation signal acts as a dominant positive for the selective activation of only the reporter harboring the p65 homodimer binding sites (35, 37, 57). We are therefore able to analyze multiple pathways for STAT3 activation by SOCS3, the formation of a protein-protein interaction between p65 homodimers and STAT3 may also be important.

Although it is unclear whether the inhibition of class 2 GAS sites is actually accomplished by competitive binding of NF-κB to overlapping GAS motifs, it has been reported that the inhibition is dependent upon proteosome (56) and p38 mitogen-activated protein kinase function (10, 11). This is consistent with a role both for NF-κB activation and serine phosphorylation of the STAT3 TAD. Our results are consistent with the involvement of NF-κB in inhibiting class 2 gene expression. However, rather than being dependent only upon competitive binding to overlapping κB sites or to the inhibition of STAT3 activation by SOCS3, the formation of a protein-protein interaction between p65 homodimers and STAT3 may also be important.

We also show that overexpression of a COOH-terminal TRAF6 MATH domain possessing an amino-terminal myristoylation signal acts as a dominant positive for the selective activation of only the reporter harboring the p65 homodimer binding site. Other reports have demonstrated a requirement for the TRAF NH2-terminal Ring and Zinc finger domains (amino acids 1–273) for NF-κB activation (57–59). However, our observation that the MATH domain can activate the p65 homodimer reporter independently parallels other reports suggesting an independent role for this domain in activating some downstream events (35, 37, 60). The MATH domain of TRAF6 is unique in that it contains a proline-rich sequence that can directly bind the SH3 domain of c-Src (35). We have confirmed this result using an in vitro protein-protein interaction assay (not shown). Although TRAF6 recruitment of c-Src may activate tyrosine kinase activity, which has been implicated in some pathways for STAT3 activation (61–64), it has also been demonstrated that for osteoclastogenesis TRAF6-mediated Src function is independent of Src kinase activity (65). Therefore, Src may be acting as an adapter molecule in downstream signaling, such as the reported activating interaction between the Src SH3 domain and a proline-rich region of the phosphatidylinositol 3-kinase p85 regulatory subunit (66). This could provide a link to the activation of NF-κB, as we (67), and others (68–70), have suggested previously.

Our TRAF6 data support the distinctness of the two types of κB binding sites; that is, they argue that the signal transduction mechanism for the two sites is different, consistent with the conclusion that the two sites bind different proteins. Also, p50/p65 heterodimer activity is known to be induced by virtually all stimuli via transforming growth factor-β-activated kinase 1 (TAK1) through IκB kinase (IKK) β targeting phosphorylation of IκBα and by several TNF family members via the NF-κB inducible kinase through IKKα to target IκBβ (71). In contrast, p65 homodimers are induced either by all known stimuli via the TAK1 pathway (like p50/p65) or by only LPS and IL-1 via unknown kinases to phosphorylate IκBβ (25). Furthermore, IκBα is induced by de novo gene expression by either IL-1 or LPS to inhibit p50/p65 hetero-, and not p65, homodimers (27). Therefore, an argument can be made that a unique signaling process for the two different NF-κB dimers may not be unreasonable. This result argues that the two different responses for the mammalian transcription factor NF-κB inducible kinase through IKKα to target IκBβ (25).

In summary, we have shown that there is an asymmetric cross-talk between STAT3 and activated NF-κB p65. This result in simultaneous STAT3 tyrosine phosphorylation-independent cooperativity on p65 homodimer binding sites and inhibition of STAT3 activation of GAS sites. Although STAT3αPTyr appears to be capable of mediating this phenomenon, it is unclear whether either IL-1 or p65 directly inhibits STAT3 tyrosine phosphorylation within the time frame that we have examined. It is possible that interaction with activated NF-κB sequesters the monomeric STAT3αPTyr, in the nucleus, away from the plasma membrane, where several JAKs function in association with the IL-6 receptor. For example, it has been reported that STAT3αPTyr monomers are constitutively imported into the nucleus and that the equilibrium toward cytoplasmic localization for STAT3 monomers in resting cells appears to be dependent upon the efficient nuclear export of STAT3αPTyr (73). Therefore, the activated p65 homodimer may function to interfere with the nuclear export of STAT3 monomers. The observation that both the IL-8 and ICAM-1 promoters, in contrast to the promoters for iNOS and Bcl-x genes, bind p65 homodimers along with STAT3 in a complex that is indistinguishable from the α2-macroglobulin-like GASκB sequence, suggests that the p50/p65-STAT3αPTyr complex may function on other genes. Therefore, the cross-talk that we observed may affect the expression of numerous genes.

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