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The Competitive Binding of STAT3 and NF-κB on an Overlapping DNA Binding Site

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Interleukin-1 (IL-1) and interleukin-6 (IL-6), two early-response cytokines expressed during an acute inflammatory reaction, regulate the expression of several acute phase proteins (APP) in the liver. IL-1 relays its signal to specific genes via NF-κB, whereas IL-6 sends its signal to the nucleus via STAT1α and STAT3. Interestingly, overlapping binding sites for STAT3 and NF-κB can be found on promoters of several APP genes. We show here that both STAT3 and NF-κB are active during inflammation and are capable of binding to a STAT3/NF-κB overlapping DNA motif derived from the α_2-macroglobulin gene promoter. *In vitro* binding assays demonstrated that NF-κB competes with STAT3 binding on this probe. Our results suggest that these transcription factors regulate each others' function through competition for overlapping DNA binding sites. © 1997 Academic Press

A network of proinflammatory mediators are activated by cell wall components of microorganisms or cellular debris of damaged tissue in the early stage of bacterial infection or tissue damage (1-3). The most prominent mediators are interleukin-1, interleukin-6 and tumor necrosis factor alpha (TNFα) (2, 3). The physiologic response of these mediators is to induce, inhibit or alter the expression of critical proteins required for host defense (3). IL-1 and TNFα activate the transcription factor NF-κB (4, 5). This factor belongs to the Rel protein family, which includes NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B, cRel and the *Drosophila* protein *Dorsal* and *Dif* (5-7). Under normal conditions NF-κB exist as an inactive form and is associated with the inhibitor protein IκB (8). Extracellular signals (IL-1, or TNFα) trigger the phosphorylation and degradation of IκB resulting in the release of NF-κB (8-14). Released NF-κB subunits form homo- and hetero-dimers that translocate to the nucleus and bind to a κB element (GGGAATTCCC), which transactivate the downstream genes (5-7, 15). On the other hand, the IL-6 signal is mediated via stimulation of cytoplasmic tyrosine kinases, called Janus kinases (JAKs), which phosphorylate latent cytoplasmic transcription factors known as STATs (16-19). In particular, IL-6 activates STAT1α and STAT3 which form hetero- and homo-dimers and translocate to the nucleus where it associates with the IL-6 responsive elements (TTC(N)nGAA) to regulate gene transcription (20-23).

In this study we explored the interaction of these two transcription factors to better understand how they may affect gene expression. Electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from rat liver undergoing an acute inflammatory response. Results show that STAT3 and NF-κB compete for overlaping DNA binding motifs with the α_2-macroglobulin gene (α2MG) promoter. Since the appearance of IL-1 precedes that of IL-6 in the inflammatory response, we propose that the transient inhibition of IL-6 responsive genes by IL-1 may be due to a competition for DNA binding between STAT3 and NF-κB.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized from Biosynthesis Inc; recombinant NF-κB1 (p50) was purchased from Promega; polyclonal antibodies to STAT3, NF-κB (p65 and p50) were purchased from Santa Cruz; monoclonal anti-phosphotyrosine antibody 4G10 was purchased from UBI; recombinant mouse IL-1β was obtained from Pfizer Inc. Polyclonal anti-fibrinogen antibodies and recombinant mouse IL-6 were produced in our laboratory (24).

Animals and cell culture. Rat primary hepatocytes were prepared and maintained in William's E media as previously described (25). For cytokine treatment, IL-1 was used at 10 ng/ml, and 100 ng/m for IL-6. Inflammed rats were produced by abdominal injection of LPS (1 mg per 500 g body weight) or injection of PBS as control.

Nuclear protein extraction. Nuclear extracts were prepared from cultured rat primary hepatocytes, or inflammatory rat liver tissue

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Abbreviations used: IL, interleukin; APP, acute phase protein; TNF, tumor necrosis factor; STAT, signal transducer and activator of transcription; LPS, lipopolysaccharide; JAK, Janus kinase; C/EBP, CAAT-enhancer binding protein; EMSA, electrophoresis mobility shift assay; IL-6RE, IL-6 responsive element.

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as described previously (25). All procedures were performed at 4°C, extracted proteins were aliquot and stored in −80°C.

Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assays were performed as previously described (25). Radiolabeled double-stranded probes were prepared by filling in the 5′ overhanging ends of reannealed complementary oligonucleotides. The various probes used in EMSAs are listed below: α2MG: derived from α_2-macroglobulin gene IL-6RE (GATCCTTCTGGGAATTCCTA) (20); SIEm67: mutant 67 of serum inducible element of c-fos gene promoter (CGACATTTCCCGTAAATCGTCG), then binds to STAT3 with high affinity (17); κB: κB site from immunoglobulin κ chain enhancer region (GATCCATGGGGAATTCCCCATG) (5); C/EBP: the CAAT enhancer binding protein (C/EBP) binding site from the IL-6 gene promoter (GACGTCACATTGCACAATCTTAATA) (26, 27). α2MGn: GATCCTTCTGGGAATTCTG, α2MGm: GATCCTTCTGGGAATTATG. For competition assays, unlabeled probes were used at 100 molar excess to radio-labeled probes. For analyses with antibodies, the nuclear extracts were preincubated with antibody (2 μg each reaction) at 4°C for 1 hour prior to EMSA procedures. For in vitro binding assay using purified NF-κB p50 (Promega), p50 protein was added in increasing amounts from 0.1 to 5 ng into control or IL-6 treated nuclear extracts, then EMSA performed. Samples were analyzed on 6% nondenaturing polyacrylamide gels in 0.5xTBE, 5% glycerol at 4°C for 2-3 hours. Dried gels were exposed to X-film at −80°C with intensifying screens.

RESULTS AND DISCUSSION

Two distinct protein complexes associate with an acute phase response element (APRE) probe during acute inflammation. Injection of lipopolysaccharide (LPS) into the peritoneal cavity of a rat induces an acute inflammatory response (20). Nuclear extracts prepared from liver tissue 1h post LPS injection were used in electrophoresis mobility shift assays (EMSA). A 20 bp oligonucleotide derived from the α_2-macroglobulin gene IL-6RE (−125 to −145) was used as the radioactive probe. Results shown in Fig 1A reveal two prominent protein/DNA complexes, (Complex I and Complex II). Since the *in vivo* LPS response can be mimicked in vitro by stimulation of hepatocytes with IL-1 and IL-6, nuclear extracts from hepatocytes treated with individual or a combination of these two cytokine were prepared for EMSA analyses. As shown in Fig 1B, IL-6 stimulation induced the formation of complex I (lane 3) while IL-1 indued complex II (lane 3). Co-stimulation with both cytokines (lane 4) mimicked the result obtained in Fig 1A.

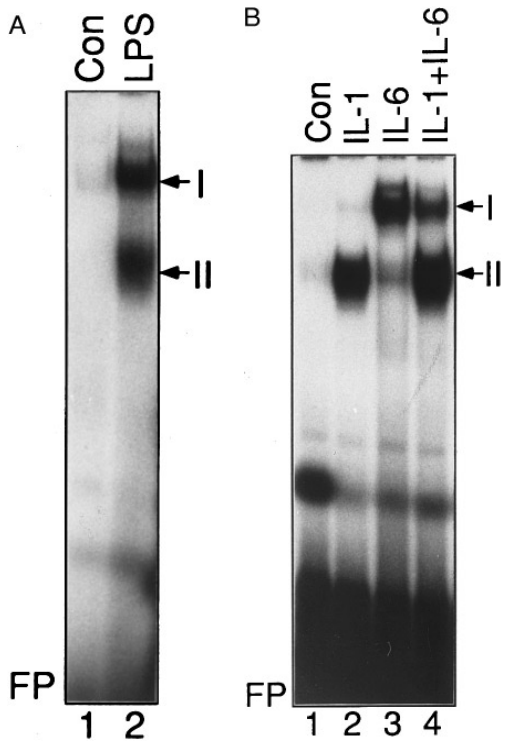
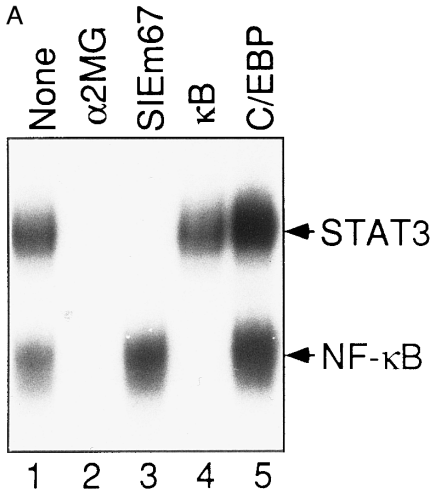


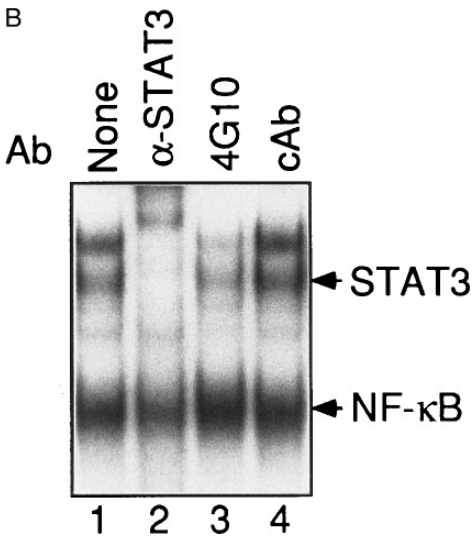
FIG. 1. Two protein complexes associate with α2MG probe during acute inflammation. EMSAs were performed as described in Materials and Methods. (**A**) Nuclear extracts were prepared from rat livers as described previously. Con, control rat; LPS, LPS injected rat. (**B**) Nuclear extracts were prepared from cultured rat primary hepatocytes treated with different cytokines. The migration positions of the two complexes are indicated as I and II. FP donates the free probes.

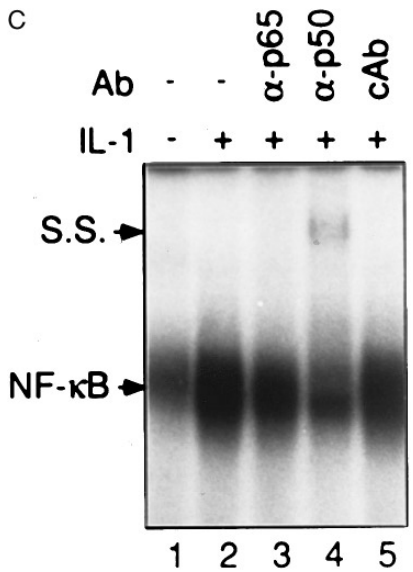
IL-1 activated NF-κB and IL-6 activated STAT3 both associate with the α2MG probe. To further analyze the two protein complexes induced by IL-1 and IL-6, competition EMSA were performed. The DNA probes used for these experiments were α2MG, SIEm67 (serum induced element), κB (kappa B) and C/EBP (CAAT/Enhancer Binding Protein) (Fig. 2A). Unlabelled α2MG probe competed with both complexes (lane2) verifying the binding specificity of the proteins to this DNA probe. Addition of the SIEm67 probe competed with complex I (lane 4) demonstrating the existence of STAT3. Addition of the κB probe competed with complex II showing it contained NF-κB. The C/EBP probe had no effect on either complex. Thus both NF-κB and STAT3 interact with the α2MG oligonucleotide as a result of IL-1 and IL-6 stimulation of hepatocytes.

The protein complexes were further characterized using specific antibodies. The addition of anti-STAT3 antibodies "super-shifted" complex I but had no effect on complex II (Fig 2B). The IL-1 induced complex (complex II) was also analyzed by the addition of antibodies to NF-κB (p65 and p50) (Fig 2C). The inclusion of anti-p65 antibodies reduced the density of the protein complex (lane 3) while anti-p50 initiated the formation of a supershifted band. Control antibodies had no effect on either complex.

The κB site overlaps with the STAT binding site. Inspection of the nucleotide sequence of the α2MG probe reveals that a putatiave NF-κB binding element overlaps with the STAT3 binding site. The binding of NF-κB to this probe was then analyzed using different length probes (increasing the C residues following the STAT3 binding site). Addition of unlabelled α2MGn probe competed the STAT3 complex but did not fully







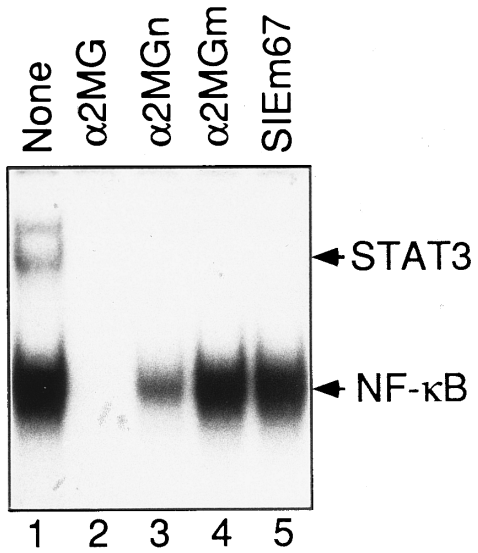


FIG. 3. NF-κB binding site overlaps with STAT3 binding site. Competition binding assays were performed as described in Materials and Methods. Different cold probes are indicated as α2MG, α2MGn, α2MGm, and SIEm67. The migration positions of STAT3 and NF-κB are indicated.

block the NF-κB complex formation (Fig 3, lane 4). Addition of unlabelled α2MGm probe prevented formation of STAT3 complex but had no effect on the NF-κB complex (lane 5). These findings indicate that the C residues following the consensus STAT3 binding site are important for NF-κB binding. NF-κB still retains binding to this DNA probe even if there is only one C residue. Recently it has been reported that activated NF-κB factors are able to associate with a κB half-site (GGGAAT) (28), while most of the IL-6REs contain this configuration.

NF-κB competes with STAT3 binding on the α2MG probe. Both NF-κB and STAT3 associate with the same 20 bp α2MG probe and it is of interest to determine whether these proteins interfere with each other for binding on their respective DNA elements. In vitro binding assays were performed using purified recombinant NF-κB1 p50 protein. Increasing concentrations of

FIG. 2. STAT3 and NF-κB both associate with α2MG probe. (**A**) Competition EMSAs. Unlabeled cold probes used for competition EMSAs are indicated as α2MG, SIEm67, κB, and C/EBP. The migration positions of STAT3 and NF-κB are indicated. (**B**) EMSAs using antibodies. The antibodies used in EMSAs are indicated as α-STAT3, polyclonal anti-STAT3 antibodies (Santa Cruz); 4G10, monoclonal anti-phosphortyrosine antibody (UBI); cAb, polyclonal anti-fibrinogen antibodies. The migration positions of STAT3 and NF-κB are indicated. (**C**) The composition of NF-κB complexes. Different antibodies are indicated as α-p65, polyclonal anti-NF-κB p65 antibodies; α-p50, polyclonal anti-NF-κB p50 antibodies (Santa Cruz); cAb, polyclonal anti-fibrinogen antibodies. The migration positions of NF-κB and the supershift band formed by anti-p50 antibodies are indicated.

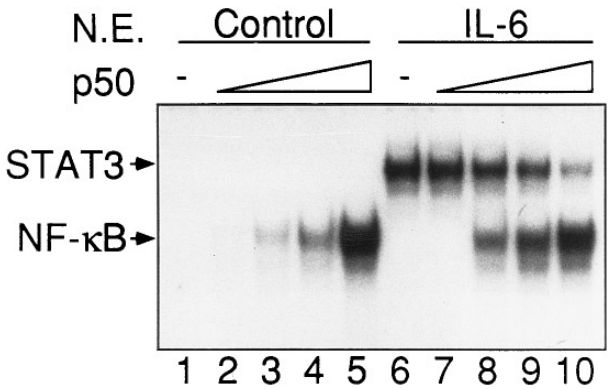


FIG. 4. NF-κB competes with STAT3 binding on the overlapping binding site. In vitro binding assays were performed as described in Materials and Methods. Recombinant NF-κB1 p50 proteins (Promega) are added at increasing amounts from 0.1 to 5 ng into control or IL-6-treated nuclear extracts. The migration positions of STAT3 and NF-κB are indicated.

NF-κB1 protein were added to binding reactions containing the α2MG probe (Fig 4, lanes 1-5). IL-6 induced STAT3 complex is shown in lane 6. As increasing amounts of p50 protein were added to the IL-6 stimulated nuclear extracts, STAT3 binding decreased (Fig 4, lanes 7-10). This finding demonstrates NF-κB effectively competes with STAT3 for association with its site, and suggests that in the case of overlapping DNA binding motifs there is insufficient space for mutual binding and that binding preference is probably related to the relative concentration of the protein within the nucleus.

Interestingly the STAT3/NF-κB overlapping sites can be found in the regulatory region of several genes (29). The first example is that of the α2-macroglobulin gene in which the native DNA sequence has a lower affinity for NF-κB and a high affinity for STAT3. The IL-6RE region on the α2MG promoter has overlapping NF-κB and STAT3 binding sites and expression of this gene can be inhibited by IL-1 (2, 3). In the case of the rat γ fibrinogen promoter there are two STAT3/NF-κB overlapping binding sites that have low binding affinity for STAT3 and high affinity for NF-κB (data not shown), and the IL-6-mediated fibrinogen gene expression is also inhibited by IL-1 (30, 31). Recently published results indicate that there is a STAT3/NF-κB binding site overlap downstream of the JunB gene (29). The consititutively association of NF-κB with this region is important for basal level expression of JunB, while STAT3 only associates with this region in response to IL-6 (29).

Together these results demonstrate that STAT3 and NF-κB which are both activated during acute inflammation associate with the α2MG gene. Furthermore NF-κB competes with STAT3 binding because of their overlapping binding elements. The observation that these two inflammatory transcription factors compete with one another for DNA binding provides a potential regulation of gene expression during an acute inflammatory reaction. Given the fact that STAT factors can be activated by various cytokines and growth factors and that all STATs bind to similar DNA motifs (18, 19, 32, 33), the current findings may offer a possible control mechanism for other cytokine-regulated genes.

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