Chapter 5

c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 Response Element (IRE)

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Summary

Transcriptional activation of eukaryotic genes often requires the cooperative action of many proteins. The Interleukin-6 (IL-6) response element (IRE) is activated by Signal transducer and Activator of transcription 3 (STAT3), and stimulation with IL-6 leads to STAT3 tyr705 phosphorylation, dimerization, translocation to the nucleus and transactivation of target gene promoters containing IREs. Here, we report that IL-6 and TPA synergistically transactivate the IRE in HepG2 cells, which is coupled to a strong upregulation of c-Jun and c-Fos expression by TPA via the MAPK pathway. Overexpression of c-Jun and c-Fos strongly enhanced STAT3 driven IRE transactivation, as well as transactivation of the human ICAM-1 promoter, while c-Jun mutants lacking the transactivation domain, the DNA binding domain or mutants in which the serine residues 63 and 73 were replaced by alanine did not cooperate with STAT3. In immunoprecipitation experiments, a direct association of STAT3 with c-Jun and c-Fos was observed in response to IL-6. Furthermore, c-Jun/STAT3 and c-Fos/STAT3 complexes were detected on IRE probes in EMSA experiments, but did not bind nor transactivate the TPA response element (TRE). These results demonstrate that AP-1 transcription factors can cooperate with STAT3 in IRE transactivation in the absence of direct AP-1 DNA binding.

Introduction

Cellular responses to extracellular stimuli are mediated by signal transduction cascades that activate specific transcription factors finally resulting in appropriate gene expression in target cells. The Signal Transducers and Activators of Transcription (STAT1-6) are a family of transcription factors involved in many vital processes in the cell [1]. STAT3 is activated in response to the Interleukin-6 (IL-6) family of cytokines including Leukemia Inhibitory Factor (LIF), IL-6, IL-11, and Oncostatin M (OnM) and is involved in proliferation and differentiation of hematopoietic cells and the induction of the acute phase response in liver cells [14,194,195,250]. Activation of STAT3 involves cytokine-induced dimerization of the gp130 receptor, association of Janus Kinases (Jaks) to the gp130 receptor complex, and JAK mediated tyrosine phosphorylation of STAT3 [1]. Tyrosine 705 phosphorylated STAT3 forms stable homodimers or heterodimerizes with STAT1 and is quickly translocated to the nucleus where it binds to specific response elements of target gene promoters [1].

c-Jun and c-Fos belong to the bZIP group of DNA binding proteins and are components of the AP-1 transcription factor complexes [263-265]. c-Jun forms homodimers or heterodimerizes with other Jun family members, including c-Fos [263]. AP-1 transcription factors bind to the TPA response elements (TRE) in promoters of target genes, and are involved in many cellular processes including proliferation, differentiation, apoptosis and stress responses [263,266]. TPA activates c-Jun via the JNK signaling cascade by inducing phosphorylation of the serine residues 63 and 73 [208,247,263]. In addition, it has been demonstrated that TPA stimulation leads to the upregulation of mRNA and protein levels of c-Jun and c-Fos [208,267]. Recently, it has been shown that a number of transcription factors can associate with AP-1 protein family members, thereby transactivating genes in a synergistic fashion. The ETS domain of the transcription factor PU.1 interacts with the basic domain of c-Jun, thus synergistically transactivating the M-CSF promoter [268].
c-Jun and c-Fos cooperate with STAT3

Smad3 can associate with both c-Jun and c-Fos in response to TGFβ, and TGFβ-mediated transcriptional activation through AP-1 sites probably involves a regulated interaction between AP-1 and Smad transcription factors [269,270]. In yeast two-hybrid experiments, an interaction between STAT3β and c-Jun has been described [124], and recently Zhang et al. mapped the interacting regions between STAT3 and c-Jun that participate in cooperative transcriptional activation [68]. Although an interaction between c-Jun and STAT3 has been demonstrated, it is still unclear whether this interaction requires direct DNA interactions of both STAT3 and c-Jun and in which manner these associations contribute to the synergism in the transactivation of genes. Independent DNA binding sites for AP-1 and STAT3 have been found in a number of gene promoters, which both contribute to gene transactivation. However, whether AP-1/STAT3 associations require DNA binding of both AP-1 and STAT3 in enhancing gene expression is presently unclear. The experiments presented here were designed to study whether c-Jun and c-Fos can interact with STAT3 and synergistically transactivate the IL-6 response element (IRE) from the human ICAM promoter in the absence of a TPA response element (TRE). We report that STAT3 can directly interact with both c-Jun and c-Fos, and that c-Jun and c-Fos cooperate with STAT3 in transactivation of the IRE in the absence of a TRE.

Materials and methods

Cell culture, reagents and antibodies

HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS, Integro B.V., Zaandam, The Netherlands) unless stated otherwise and U937 cells were grown in RPMI supplemented with 10% FCS. Cells were stimulated with 10 ng/ml human recombinant IL-6 (generous gift from Dr. S.C. Clark, Genetics Institute, Cambridge, USA) or 100 ng/ml TPA (Sigma). Antibodies against STAT3 (C-20), c-Jun (H-79), c-Fos (4) and ERK-1 (K-23) (Santa Cruz) were used in dilutions of 1:4000. Antibodies against phosphorylated STAT3 (Tyr705) and ERK (Thr202/Tyr204) were obtained from New England Biolabs and used in a 1:1000 dilution. Supershift antibodies against STAT3 (C-20X), c-Jun (H-79X) and c-Fos (4X) were obtained from Santa Cruz and 1 µl was used in each experiment. The MEK inhibitor PD98059 (Santa Cruz) was used at a final concentration of 20 µM and was added to the cells 30 min prior to stimulation with IL-6 or TPA.

Expression and reporter constructs

The pIRE-ti-LUC reporter was made by inserting a synthetic oligonucleotide (5’-ctagcaggTTTCCGGGAAAgcacagcttaggTTTCCGGGAAAgcac-3’) containing two copies of the IL-6 response element (IRE) of the ICAM-1 promoter in the NheI site of the pGL3ti minimal promoter luciferase construct [246]. Similar, pIREmut-ti-LUC was constructed by using a synthetic oligonucleotide (5’-ctagcaggTTAGCGGTCAAgcacagcttaggTTAGCGGTCAAgcac-3’) with mutated IRE sites. The pIC-1014-LUC reporter containing a 1014 bp fragment of the human ICAM-1 promoter and the pIC-1014(IRE-mut)-LUC reporter in which the IRE site was mutated were previously described [224]. pTRE-ti-LUC was constructed by inserting a synthetic oligonucleotide (5’-gatctcgcttgatgAGTCAGcgcgaag-3’) with a TRE site in BglII-BamHI sites of the pGL3ti reporter and pTREMUT-ti-LUC by inserting an oligonucleotide with a mutated TRE site (5’-gatctcgcttgatgAGTTGGccggaag-3’) in the BglII-BamHI sites of pGL3ti. Constructs were
verified by sequencing. Furthermore, the following eukaryotic expression vectors were used: pRSV-c-Jun, encoding human c-Jun, and pRSV-c-Fos, encoding human c-Fos (both kindly provided by Dr. H. van Dam, Dep. of Medical Biochemistry, RUL, Leiden, The Netherlands); pRSV-c-Jun(6-223) which lacks the transactivation domain from the residues 6 to 223, pRSV-c-Jun(ΔDBD), which lacks the DNA binding domain, and pRSV-c-Jun (ser63/73ala) in which the serine residues 63 and 73 were replaced by alanine residues (kindly provided by Dr. P. Angel, Institute of Genetics, Forschungszentrum Karlsruhe, Karlsruhe, Germany); and pSG5-STAT3, encoding human STAT3α (generous gift from Dr. P. Coffer, Dep. of Pulmonary Diseases, AZU, Utrecht, The Netherlands).

**Transient transfections**

HepG2 cells were seeded at 3x10⁵ cells per well in 6-well plates (Costar), and 24 hours later cells were transfected with 10 µg plasmid DNA using the calcium phosphate co-precipitation method [230]. Transfection mixtures consisted of a mixture of 3 µg pIRE LUC reporter, 3 µg pDM2-LacZ as a control to determine transfection efficiency, and 4 µg of expression plasmids as mentioned in the results section. When necessary, pUC18 was added to the transfection mixture to obtain a total of 10 µg of DNA. Cells were incubated with precipitate for 24 hours, washed with phosphate buffered saline (PBS), and stimulated for an additional 24 hours. Cells were collected in 200 µl reporter lysis buffer (Promega) and subjected to the assays for luciferase [231] and β-galactosidase [232] as previously described. The data represent two independent experiments using different batches of DNA, and in each experiment transient transfections were performed in triplicate. Standard deviations were calculated using Sigmaplot (Jandel Corp.).

**SDS-polyacrylamide gel electrophoresis, Western blotting and immunoprecipitations**

A total of 1x10⁷ cells were lysed on ice in lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM DTT, 1 mM Na₂VO₃ (ortho), 1% Triton X100, 10% glycerol, 10 µg/ml leupeptin, and 0.4 mM PMSF). Protein concentrations were determined (Biorad). Whole-cell extracts were boiled for 5 min in the presence of Laemmli sample buffer prior to separation on 12.5% SDS-polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (Millipore) in Tris-glycine buffer at 100 Volts for 1.5 h using an electroblotter (Pharmacia). Membranes were blocked with PBS buffer containing 5% non-fat milk prior to incubation with antibodies. Binding of each antibody was detected by chemiluminesence using ECL according to the manufacturer’s recommendations (Amersham Corp.). For immunoprecipitations, whole cell lysates were incubated with antibodies, precipitated with Protein-A Sepharose beads (Pharmacia), and washed two times with lysis buffer. The precipitates were boiled for 5 min in Laemmli sample buffer and subjected to 12.5% SDS-polyacrylamide gel electrophoresis.

**EMSA**

Nuclear extracts were prepared from 10⁷ cells according to the rapid Dignam method as described previously [271]. Double-stranded synthetic oligos comprising the IL-6RE (IRE) (upper strand: 5’-cgcgtagctaggTTTCGGGAAAgcgagc-3’), the TPA response element (TRE) (upper strand: 5’-gatctcgcttgatgAGTCAGgccgaag-3’), or the mutated TPA response element (TREMUT) (upper strand: 5’-gatctcgcttgatgAGTTGGccggaag-3’) were ³²P-labeled by filling in the 5’-protruding ends with γ³²P-dATP and Klenow enzyme. Binding reactions contained 5 µg of nuclear extract, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 20 mM HEPES, pH 7.9, 100 ng of poly(dI-dC) and
approximately 10,000 cpm labeled probe and 100-fold molar excess of competitor oligonucleotide where appropriate. Protein binding was allowed to proceed for 30 min at room temperature. Then 20% Ficoll was added to the reactions, and samples were immediately loaded onto 5% polyacrylamide gels containing 0.5× TBE. In supershift experiments, Stat3, c-Jun and c-Fos antibodies (Santa Cruz) were added to the nuclear extract 15 min prior to incubation with labeled probe.

Results

TPA upregulates c-Jun and c-Fos expression in HepG2 cells and enhances the transcriptional activity of the pIRE-ti-LUC reporter. It has been demonstrated that the phorbol ester TPA is capable of strongly upregulating c-Jun and c-Fos expression [208,267]. Since AP-1 transcription factors have been implicated in the cooperative transactivation of gene promoters in cooperation with STAT3 [68,124], we decided to study whether TPA stimulation could enhance IRE transactivation by inducing c-Jun and

Figure 1. TPA upregulates c-Jun and c-Fos expression in HepG2 cells and enhances the IL-6 induced transcriptional activity of the pIRE-ti-LUC reporter. A, HepG2 cells were transiently transfected with pIRE-ti-LUC reporters and stimulated with 10 ng/ml IL-6, 100 ng/ml TPA or both for 24 hrs as indicated, followed by luciferase and LacZ assays. Where indicated, cells were preincubated with 20 µM PD98059 for 60 min. B, HepG2 cells were serum starved in 0.5% FCS (lanes 1-10) or cultured in 10% FCS (lane 11), stimulated with 10 ng/ml IL-6 (lanes 2-4) or 100 ng/ml TPA (lanes 5-7) for 1-3 hrs, or transfected with pRSV-c-Fos (lane 9) or pRSV-c-Jun (lane 10). In lane 8, HepG2 cells were preincubated with 20 µM PD98059 prior to stimulation with TPA. Total cell lysates were Western blotted and analyzed for c-Jun, c-Fos and STAT3 expression. In the bottom panel, the effects of TPA stimulation and PD98059 pretreatment on ERK phosphorylation is shown. C, HepG2 cells were stimulated with IL-6, TPA, or both as indicated for 15 min and total cell lysates were Western blotted and analyzed for STAT3 tyr705 phosphorylation. As a control, blots were stripped and reprobed for STAT3. D, Nuclear extracts were prepared from unstimulated or IL-6 and/or TPA stimulated HepG2 cells (15 min) as indicated. EMSAs were performed using a labeled IRE probe.
c-Fos expression. HepG2 cells were transiently transfected with a pIRE-ti-LUC reporter containing two STAT3 binding sites from the human ICAM promoter and stimulated with IL-6 and/or TPA. As depicted in figure 1A, IL-6 stimulation resulted in a 4.9 fold induction of the IRE, while TPA stimulation also resulted in a slight induction of IRE transactivation levels of 2.6-fold. Co-stimulation with both IL-6 and TPA strongly enhanced IRE transactivation 17.4 fold over unstimulated levels (Fig.1A). Inhibition of MEK activity by treating HepG2 cells with the inhibitor PD98059 (20 µM) resulted in a loss of the TPA response, while the IL-6-induced IRE transactivation was unaffected (Fig.1A). To confirm that TPA indeed upregulates c-Jun and c-Fos expression in HepG2 cells, cells were stimulated with TPA and total cell lysates were examined for c-Jun and c-Fos expression. Indeed, TPA strongly upregulated c-Jun and c-Fos expression within 1 hour in cells cultured in 0.5% FCS, comparable to the c-Jun and c-Fos overexpression levels (Fig.1B, lanes 5-7 and 9-10). In HepG2 cells cultured in 10% FCS, low basal levels of c-Jun and c-Fos expression were observed (Fig.1B, lane 11) which were further upregulated by TPA treatment (data not shown). This upregulation was completely blocked by PD98059 pretreatment, indicating that TPA upregulates c-Jun and c-Fos expression via the MAPK pathway in HepG2 cells (Fig.1B, lane 8). To further confirm that TPA induces ERK activation, HepG2 cells were stimulated for 20 min with 100 ng/ml TPA and total cell lysates were Western blotted using antibodies against phosphorylated ERK-1. As depicted in Fig.1B, TPA induced ERK phosphorylation within 20 min, which was completely blocked by PD98059 pretreatment. In contrast to the results obtained with TPA, IL-6 did not upregulate c-Jun or c-Fos expression (Fig.1B, lanes 1-4).

To exclude the possibility that TPA directly influences IL-6-induced STAT3 signaling when administered to the cells at the same time, the effects of TPA and IL-6 on STAT3 tyr705 phosphorylation and DNA binding were studied. As depicted in Fig.1C and D, co-stimulation with both TPA and IL-6 neither modulated the IL-6-induced STAT3 tyr705 phosphorylation nor DNA binding, while TPA alone did not induce STAT3 tyr705 phosphorylation or DNA binding. These results indicate that the TPA effects on IRE transactivation do not involve direct modifications of STAT3. Taken together, these results indicate that the TPA enhanced IRE transactivation coincides with a strong upregulation of c-Jun and c-Fos expression.

**C-Jun and C-Fos overexpression strongly enhance IL-6-induced IRE transactivation.**

To further study the effects of c-Jun and c-Fos on IL-6-induced STAT3 transactivation, c-Jun and c-Fos were transiently overexpressed in HepG2 cells together with the pIRE-ti-LUC reporter. IL-6-induced a 5.0-fold induction of the reporter gene, and overexpression of c-Jun or c-Fos strongly enhanced both basal and IL-6-induced STAT3 transactivation (Fig.2A). The IL-6 dependent responses in the presence of overexpressed c-Jun or c-Fos were about 25-fold and 16-fold higher than that of the uninduced reporter without c-Jun or c-Fos overexpression. As a control, a reporter with mutated IRE sites (pIREmut-ti-LUC) was transiently transfected together with expression vectors for c-Jun and c-Fos. IL-6 stimulation did not activate this reporter, and no enhanced transactivation was detected in the presence of overexpressed c-Jun or c-Fos (Fig.2A). We observed a basal IRE transactivation in unstimulated cells which was reduced with the IREmut reporter (Fig.2A), indicating that there is some basal STAT3 transactivation in the absence of IL-6 stimulation. Overexpression of c-Jun and c-Fos increased unstimulated IRE transactivation.
levels (Fig.2A), suggesting that c-Jun and c-Fos can cooperate with this basal STAT3 activity.

Figure 2. Overexpression of c-Jun or c-Fos enhances the IL-6 induced IRE transactivation. A, HepG2 cells were transfected with pRSV-c-Jun, pRSV-c-Fos, and pIRE-ti-LUC or pIREmut-ti-LUC reporters as indicated. Cells were stimulated with 10 ng/ml IL-6 for 24 hrs, followed by luciferase and LacZ assays. B, Transient transfection as in A, but now cells were transfected with the pIRE-ti-LUC reporter, together with pRSV-c-Jun, pRSV-c-Jun (6-223), pRSV-c-Jun (ΔDBD) or pRSV-c-Jun (ser63/73ala). C, Transient transfection as in A, but now cells were transfected with the pIC-1014-LUC or pIC-1014(IREmut)-LUC reporter as indicated, together with pRSV-c-Jun, pRSV-c-Jun (6-223), pRSV-c-Jun (ΔDBD) or pRSV-c-Jun (ser63/73ala). Where indicated, cells were co-stimulated with 100 ng/ml TPA. D, HepG2 cells were transfected with pRSV-c-Jun, and pTRE-ti-LUC or pTREmut-ti-LUC reporters as indicated. Cells were stimulated with 10 ng/ml IL-6, 100 ng/ml TPA or both for 24 hrs as indicated.

To investigate the structural requirements of the interaction between STAT3 and AP1 proteins, c-Jun mutants lacking the transactivation domain (Δ6-223), the DNA binding domain (ΔDBD) or c-Jun mutants in which the serine residues 63 and 73 were replaced by alanine residues were overexpressed in HepG2 cells and the effects on IL-6-induced IRE transactivation were studied. Overexpression of either of these mutants did not enhance IRE transactivation (Fig.2B), suggesting that a full-length c-Jun protein is required for cooperating with STAT3.
Furthermore, we investigated whether c-Jun and c-Fos also cooperate with STAT3 in activating full-length IL-6 responsive promoters. Reporters containing either the human ICAM-1 promoter (pIC1014-LUC) or the human ICAM-1 promoter in which the IRE site was mutated (pIC1014(IREmut)-LUC) [224] were transfected in HepG2 cells together with expression vectors for c-Jun, c-Fos or the mutant c-Jun (ser63/73ala). As depicted in Fig.2C, the IL-6-induced reporter activation was significantly enhanced in the presence of overexpressed c-Jun or c-Fos, while overexpression of c-Jun (ser63/73ala) did not modulate the IL-6-induced reporter activation. Also, co-stimulation with both IL-6 and TPA resulted in enhanced reporter activity as compared to stimulation with IL-6 alone (Fig.2C). The pIC1014(IREmut)-LUC reporter was not induced by overexpression of c-Jun or c-Fos, or by treatment with IL-6 and/or TPA. Taken together, these data indicate that c-Jun and c-Fos can cooperate with STAT3 in transactivating the IRE of IL-6 responsive promoters.

To determine whether AP-1/STAT3 complexes can also transactivate the TRE, HepG2 cells were transiently transfected with a pTRE-ti-LUC reporter, with or without expression vectors for c-Jun. As depicted in Fig.2D, stimulation with IL-6 did not alter the transactivation of the pTRE-ti-LUC reporter. As a positive control, cells were stimulated with TPA, which strongly enhanced transactivation (Fig.2D). Stimulation with both IL-6 and TPA did not further enhance the TPA-induced transactivation of the TRE (Fig.2D). Overexpression of c-Jun enhanced TPA-induced transactivation of the pTRE-ti-LUC reporter, but activation of STAT3 by stimulating cells with IL-6 did not alter TRE transactivation (Fig.2D). As a control, cells were transiently transfected with the pTREmut-ti-LUC reporter, which was unresponsive to c-Jun overexpression or stimulation with TPA and IL-6 (Fig.2D). To further confirm that STAT3/AP-1 complexes did not bind to the TRE, EMSAs were performed using a labeled TRE probe and nuclear extracts from HepG2 cells. Supershift analysis demonstrated that STAT3 was not present in complexes on the TRE when stimulated with IL-6 and/or TPA (data not shown). Taken together, these results demonstrate that AP-1/STAT3 complexes can neither bind nor transactivate the TRE.

**c-Fos and c-Jun co-immunoprecipitate with STAT3.** To explore whether STAT3 can directly interact with AP-1 proteins, HepG2 cells were transiently transfected with either c-Jun or c-Fos. Cells were either left unstimulated or stimulated with IL-6 for 15 min and nuclear extracts were isolated. c-Jun and c-Fos were immunoprecipitated and immunoprecipitates were blotted against STAT3. As depicted in Fig.3A, STAT3 co-immunoprecipitated with both c-Jun and c-Fos upon IL-6 stimulation, whereas in unstimulated cells no interaction between c-Jun and STAT3 or c-Fos and STAT3 could be detected. As a control, the levels of overexpressed c-Jun and c-Fos are shown (Fig.3A, lower panels).

Reversibly, STAT3 was immunoprecipitated from nuclear extracts of HepG2 cells in which c-Jun and c-Fos were overexpressed. The immunoprecipitates were Western blotted using antibodies against c-Jun and c-Fos. Again, both c-Jun and c-Fos co-immunoprecipitated with STAT3 upon IL-6 stimulation (Fig.3B, upper panels). As a control, blots were stripped and reprobed against STAT3 (Fig.3B, middle panels). Also, total nuclear extracts were Western blotted against STAT3 and c-Jun/c-Fos. Overexpressed c-Jun and c-Fos were present in both unstimulated as well as IL-6-
stimulated cells, whereas STAT3 only localized in the nuclear fractions upon IL-6 stimulation (Fig.3B, lower panels). Taken together, these data demonstrate that STAT3 can directly associate with c-Fos and c-Jun.

**Figure 3. c-Jun and c-Fos co-immunoprecipitate with STAT3.** A, HepG2 cells were transfected with pRSV-c-Jun or pRSV-c-Fos, stimulated with IL-6 for 15 min as indicated and nuclear extracts were prepared. c-Jun and c-Fos were immunoprecipitated using and immunoprecipitates were Western blotted against STAT3. As a control, the overexpression levels of c-Jun and c-Fos are shown (bottom panels). B, Immunoprecipitation as in A, but now STAT3 was immunoprecipitated and co-immunoprecipitated c-Jun and c-Fos were analyzed by Western blotting. As a control, blots were stripped and reprobed using anti-STAT3 antibodies (middle panels). Furthermore, Western blots of total nuclear extracts are shown using antibodies against STAT3, c-Jun and c-Fos (bottom panels).

c-Jun and c-Fos are present in complexes bound to the IRE. To determine whether there exists a physical interaction between STAT3 and AP-1 proteins on the IRE, Electrophoretic Mobility Shift Assays (EMSAs) were performed using a labeled IRE probe and nuclear extracts from unstimulated or HepG2 cells stimulated with IL-6 for 15 min. Stimulation with 10 ng/ml IL-6 induced strong binding of the STAT3/3 homodimer
to the IRE, although low levels of STAT1/1 homo- and STAT1/3 heterodimers were also observed (Fig. 4, lanes 1-2). In a competition experiment using a 100 fold molar excess cold IRE, all bands were effectively competed (Fig. 4, lanes 1-3). In the supershift experiments using anti-STAT3 antibodies, the STAT1/3 heterodimer and the STAT3/3 homodimer were supershifted (Fig. 4A, lane 4-6). Furthermore, supershift experiments were performed using antibodies against c-Fos and c-Jun. Pretreating nuclear extracts with ant-c-Jun or anti-c-Fos antibodies strongly reduced STAT3/3 DNA binding, indicating that c-Jun and c-Fos are present in this IRE bound complex (Fig. 4, lanes 7-10). Also, diminished STAT1/1 and STAT1/3 DNA binding was observed. Since no supershifted band could be detected, these data suggest that binding of antibodies to c-Jun or c-Fos prevent the association of the complexes to the DNA probe.

![Figure 4](image.png)

**Figure 4. c-Jun and c-Fos are present in complexes bound to the IRE.** Nuclear extracts were prepared from unstimulated or IL-6 stimulated HepG2 cells (10 ng/ml, 15 min) as indicated. EMSAs were performed using a labeled IRE probe, competition experiments with 100 molar excess cold IRE probe and supershift experiments using 1 µl of antibodies against STAT3, c-Jun, or c-Fos as indicated.

**Discussion**

It is now increasingly becoming clear that transcriptional activation of eukaryotic genes requires the cooperative function of several proteins [272]. Here, we provide evidence for a functional and direct association between STAT3 and AP-1 transcription factors in transactivation of the IRE. In immunoprecipitation experiments in HepG2 cells, STAT3-c-Jun and STAT3-c-Fos interactions were detected in unstimulated cells as well as in IL-6 stimulated cells. In EMSAs we find that c-Jun and c-Fos are present in complexes that bind the IRE, and overexpression of c-Jun or c-Fos strongly enhances IRE driven transcription.
A number of gene promoters that contain both AP-1 (TRE) and STAT3 (IRE) binding sites have now been identified, including the junB [147], vasoactive intestinal peptide [144], the c-Fos [145], and the α₂-macroglobulin promoters [146]. In these cases, it has been demonstrated that both the STAT3 and AP-1 DNA binding sites contribute to the regulated expression of these genes. Furthermore, recent evidence indicated that STAT3 can directly associate with c-Jun, and that STAT3 and c-Jun participate in cooperative transcriptional activation of the α₂-macroglobulin enhancer, which contains both STAT3 and AP-1 response elements [68]. In addition, recent studies have indicated that AP-1 can also directly associate with a number of transcription factors in the absence of a TRE, including the transcription factor PU.1 [268]. The ETS domain of the transcription factor PU.1 interacts with the basic domain of c-Jun, thus synergistically transactivating the M-CSF promoter [268]. In this enhanceosome, PU.1 functions as a DNA binding factor and possibly the function of c-Jun is to stabilize the complex, or to recruit co-factors to enhance transactivation without directly binding the DNA [268]. Similarly, we find that c-Jun and c-Fos can directly associate with STAT3. In immunoprecipitation experiments in HepG2 cells both c-Jun and c-Fos were co-immunoprecipitated with STAT3 in response to IL-6. Reversely, STAT3 was co-immunoprecipitated with c-Jun and c-Fos in IL-6 stimulated cells.

Our experiments indicate that c-Jun and c-Fos are present in complexes that bind to the IRE in EMSA supershift experiments using antibodies against c-Jun and c-Fos. Since AP-1 transcription factors do not bind directly to the IRE, we hypothesize that STAT3 is the DNA binding factor in this complex, and that c-Jun and/or c-Fos associate with STAT3 without directly binding to the DNA. In contrast to supershift experiments using antibodies against STAT3, we did not observe supershifted complexes when antibodies against c-Jun or c-Fos were used. Apparently, binding of antibodies against c-Jun or c-Fos prevents the association of the complexes to the IRE probe. Indeed, it has been suggested that one of the c-Jun interacting domains of STAT3 is located in the DNA binding region [68], thus possibly accounting for the observed results. Furthermore, also a diminished STAT1/1 DNA binding was observed, although previous reports have suggested that c-Jun does not interact with STAT1 [68]. Whether AP-1 proteins also cooperate with STAT1 in transactivating genes remains to be elucidated.

Importantly, overexpression of c-Jun or c-Fos strongly enhanced IRE driven transcription, while the luciferase reporter containing mutated IRE sites was not induced by c-Jun or c-Fos overexpression. Also, the activity of the full-length IL-6 responsive human ICAM-1 promoter was enhanced in the presence of overexpressed c-Jun or c-Fos. These data confirm the model in which IL-6 stimulation induces STAT3/3 homodimerisation and translocation to the nucleus, followed by DNA binding and STAT3-c-Jun or STAT3-c-Fos association, which strongly enhances transcriptional activation. In this model, direct c-Jun or c-Fos DNA binding is not required for cooperation with STAT3. The possibility of a STAT3-AP-1 cooperation in TRE transactivation can be excluded, since no STAT3 was found in complexes that bind the TRE in response to IL-6 and TPA (data not shown). Moreover, TRE-LUC reporter activation was not enhanced by IL-6-activated STAT3.

Recently, Zhang et al. described the regions of interaction in STAT3 and c-Jun that participate in cooperate transcriptional activation [68]. In in vitro pull down assays, they located a segment of STAT3 from residues ~130-358 which binds to the C-terminal part of c-Jun. To identify the important regions of c-Jun involved in cooperatively transactivating the IRE, c-Jun mutants lacking the transactivation domain (residues 6-233) or the DNA binding domain were overexpressed in HepG2 cells together with the IRE reporter.
Overexpression of either of these mutants failed to enhance STAT3 driven IRE transactivation, indicating that these functional domains in c-Jun are required for the cooperative action with STAT3. Furthermore, the c-Jun (ser63/73ala) mutant did not cooperate with STAT3, suggesting that serine phosphorylation of c-Jun is also required. Behre et al. recently demonstrated that the c-Jun transactivation domain, the basic domain and the leucine zipper domain are all required for the activation of PU.1 by c-Jun, although the basic domain is probably the domain of interaction with the Ets domain of PU.1 [268]. In contrast, JNK-1-induced ser63 and ser73 phosphorylation of c-Jun is not important for its coactivation function for PU.1 [268]. Whether serine phosphorylation in the c-Jun activation domain is required for the synergistic action with STAT3 requires further experiments.

The cooperative action of STAT3 and AP-1 proteins to modulate the degree of transactivation provides the possibility to fine-tune the expression of downstream target genes in response to a variety of growth factors. Indeed, we find that upregulation of c-Jun and c-Fos expression, by treating the cells with TPA, strongly enhances activation of the IRE reporter, while TPA does not affect STAT3 tyrosine705 phosphorylation or DNA binding. In agreement with the results obtained in the IRE transactivation assays, in which c-Jun or c-Fos overexpression enhanced basal IRE transactivation, TPA alone also induced a significant IRE transactivation in the absence of IL-6. This is probably the resultant of TPA-induced upregulation of c-Jun and/or c-Fos expression, which then cooperates with basal STAT3 transactivation. In contrast to these findings, it has been reported that activation of the MAPK pathway by TPA negatively influences STAT3 tyr705 phosphorylation and transactivation if cells are pretreated with TPA prior to IL-6 stimulation [139,273-275]. However, we clearly observed no TPA effects on IL-6-induced STAT3 DNA binding or tyr705 phosphorylation in HepG2 cells when both stimuli were administered to the cells simultaneously. Probably, pretreatment is essential for the TPA-induced downregulation of IL-6-induced STAT3 activation.

The observed cooperative function of IL-6 and TPA indicate that different signaling pathways affect the degree of STAT3 transactivation. In HepG2 cells, the IL-6-induced STAT3 transactivation and ser727 phosphorylation is mediated by a signaling cascade that involves gp130, Vav, Rac, MEKK and SEK/MKK-4 [241]. The TPA-induced activation of the IRE is mediated via the MAPK pathway and is correlated to a strong upregulation of c-Jun and c-Fos proteins. Cooperative activation of both pathways leads to a synergistic effect on the STAT3 transactivation.

Taken together, we demonstrate that c-Jun and c-Fos can associate with STAT3 and strongly enhance IRE driven transcription in the absence of TPA response elements. It will be challenging to determine the importance of these AP-1/STAT3 associations during target gene regulation in relevance to cellular processes including proliferation, differentiation or apoptotic events as well as cellular transformation.