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Amino Acid Residues Required for Physical and Cooperative Transcriptional Interaction of STAT3 and AP-1 Proteins c-Jun and c-Fos▽

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Cooperation between STAT3 and c-Jun in driving transcription during transfection of reporter constructs is well established, and both proteins are present on some interleukin-6 (IL-6) STAT3-dependent promoters on chromosomal loci. We report that small interfering RNA knockdown of c-Jun or c-Fos diminishes IL-6 induction of some but not all STAT3-dependent mRNAs. Specific contact sites in STAT3 responsible for interaction of a domain of STAT3 with c-Jun were known. Here we show that the B-zip domain of c-Jun interacts with STAT3 and that c-Jun mutation R261A or R261D near but not in the DNA binding domain blocks in vitro STAT3-c-Jun interaction and decreases costimulation of transcription in transfection assays. Cooperative binding to DNA of tyrosine-phosphorylated STAT3 and both wild-type and R261A mutant c-Jun was observed. Even c-Jun mutant R261D, which on its own did not bind DNA, bound DNA weakly in the presence of STAT3. We conclude that a functional interaction between STAT3 and c-Jun while bound to chromosomal DNA elements exists and is necessary for driving transcription on at least some STAT3 target genes. Identifying such required interactive protein interfaces should be a stimulus to search for compounds that could ultimately inhibit the activity of STAT3 in tumors dependent on persistently active STAT3.

Latent transcription factors are often the ultimate agents of carcinogenesis when signaling pathways are dysregulated (8). Thus, overactive NF-κB, GLI proteins, Notch NICD, β-catenin, and STAT3 and -5 have all been implicated in human cancer. While interruption of this overactivity theoretically offers multiple therapeutic target opportunities (blocking receptors, proteases, kinases, nuclear accumulation, etc.), the most direct means of inhibition would be inhibition of the activity of the transcription factor itself. Because no success at specific in vivo inhibition of DNA binding of a single target factor has ever been achieved practically, direct inhibition of a target transcription factor may well involve blocking a required protein interaction between the targeted transcription factor and another nuclear protein. In fact two instances of such specific inhibition, compounds that interrupt myc-max (5, 30) or p53-MDM associations (16), have been reported.

The STATs are latent transcription factors activated by cytoplasmic tyrosine kinases (18). Normally STAT activation is transient, which is assured by a variety of negatively acting events that block further activation, decrease DNA binding, or result in dephosphorylation of STAT3 (1, 25). STAT3 is persistently active in a wide variety of human solid tumors as well as leukemia and lymphomas (32). Moreover, cell lines from such tumors show a requirement for continued STAT3 activation to grow and/or to resist apoptosis. Interruption of persistent STAT3 activation by dominant negative proteins, by "decoy" homologues of DNA binding sites, by kinase inhibitors, and most recently by compounds that inhibit STAT3 activity through as yet unknown mechanisms has been reported (9, 19, 32).

We have studied the cooperation of STAT3 with other proteins in driving transcription with the aim of learning about specific protein interactions that could serve as targets for interruption of activated STAT3 activity. STAT3 (in fact STAT3β, usually considered a dominant negative STAT3 isoform) and c-Jun were first reported to cooperate in driving transcription by Schaefer et al. (24). We later showed an in vitro interaction between the coiled-coil domain of STAT3 and a large COOH-terminal segment of c-Jun (33). Moreover, the c-Jun protein was found to be constitutively present on the promoter of a well-defined STAT3-induced gene (the α2-macroblobulin [α2-M] gene) prior to the arrival of phosphorylated STAT3 after interleukin-6 (IL-6) gene activation (17).

We have extended the study of STAT3-c-Jun cooperation in the present work by identifying the requirement of c-Jun and c-Fos for α2-M induction as well as locating individual residues in c-Jun that are required both for in vitro interaction between the proteins and for maximal transcriptional induction of reporter constructs.

METHODS AND MATERIALS

Tissue culture. Rat hepatoblastoma (H35) cells were cultured (9% CO_2, 37°C) in Dulbecco's modified Eagle's medium (Gibco), supplemented with a 100× penicillin-streptomycin mixture (Gibco), 5% fetal bovine serum (Gibco), and 20% horse serum (BioWhittaker). HepG2 cells were cultured in Eagle minimum essential medium (ATCC), supplemented with 100× penicillin-streptomycin mixture (Gibco), 100× antibiotic-antimycotic mixture (Gibco), and 10% fetal bovine serum (Gibco). 293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco), supplemented with 100× penicillin-streptomycin mixture (Gibco) and 10% fetal bovine serum (Gibco). For mRNA induction by IL-6 and

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dexamethasone treatment, cells were starved with low-serum medium (overnight with 1% fetal bovine serum).

Reagents and antibodies. Human IL-6 and human IL-6 receptor (R&D Systems) were used at concentrations of 80 ng/ml and 100 ng/ml, respectively. Dexamethasone (Sigma) was diluted in ethanol and used at a final concentration of 100 nM. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA; Sigma) was used at a final concentration of 0.1 ng/ml. Oncostatin M (OSM; R&D Systems) was used at a final concentration of 20 ng/ml. Antibodies for supershift and Western blot analysis were purchased from Abcam (anti-c-Jun and anti-c-Fos), Cell Signaling (anti-phospho-c-Jun Ser 63), Santa Cruz [anti-c-*myc*, anti-STAT3 (c-term)], and Ambion (anti-GAPDH [glyceraldehyde-3-phosphate dehydrogenase]).

Plasmids. The pRSV-cJun mammalian expression vector was a gift from Daniel Besser (Rockefeller University). The SOCS-3 luciferase reporter (−159 to +929: clone 6T3) was a gift from Shlomo Melmed (Cedars Sinai Research Institute, Los Angeles, CA). The α2-M luciferase reporter (−1151 to +54) was a gift from George H. Fey (University of Erlangen-Nueremberg, Erlangen, Germany). The truncated α2-M luciferase reporter (−200 to +54) was a gift from Daniel Nathans (Johns Hopkins University), from which the α2-M luciferase reporter (−200 to −100) was generated. Specific mutations in these α2-M luciferase plasmids were generated in this lab and have been so described previously (17). The glutathione *S*-transferase (GST) STAT3 plasmids were also constructed in the Darnell lab and have been previously described. (33). The cytomegalovirus-*Renilla* luciferase plasmid was purchased from Promega. Myc-GST-tagged c-Jun and c-Fos plasmids were created by first cloning the full gene (flanked by EcoRI and XhoI sites) into the pCMV-myc plasmid (BD-Bioscience). GST (amino acids 1 to 221) and a factor Xa cleavage site were then cloned (from the pGEX-5X-1 plasmid) into our constructs downstream of the myc tag and upstream of the full gene (Jun or Fos gene) via EcoRI. All constructs were confirmed via sequencing (Genewiz, Inc.).

Site-directed mutagenesis. The QuikChange II-XL site-directed mutagenesis kit was used to make specific point mutations in pRSV-cJun and -cFos plasmids (Stratagene). The following primer sets were used (only the 5′ strands are described; these sequences, along with 3′ complementary strands, were produced and purchased from Fisheroligo): Jun E256A, 5′-CGGATCAAGGCGGCGAG GAAGCGC-3′; Jun E256A,R257A, 5′-CGGATCAAGGCGGCGGCGAAGC GCATGAAG-3′ (made from E256A mutant); Jun M260A, 5′-GAGAGGAAG CGCGCGAGGAACCGCATC-3′; Jun M260A,R261A, 5′-GAGAGGAAGCG CGCGGCGAACCGCATCGCT-3′ (made from M260A mutant); Jun 4MUT, 5′-GCGGCGAAGCGCGGCGAACCGCATCGCT-3′ (made from E256A, R257A mutant); Jun R261A, 5′-GAGAGGAAGCGCATGGCGAACCGCATCGCT-3′; Jun R261D, 5′-GAGAGGAAGCGCATGGACAACCGCATCGCT-3′; Fos R146A, 5′-AGAATCCGAAGGGAAGCGAATAAGATGGCT-3′; Fos R146D, 5′-AGAATCCGAAGGGAAGACAATAAGATGGCT-3′; Jun S63A, 5′-ACCG CGGCCCGACGTGGGGCTGCTCAAGCTGGCGGCGCCC. Only essential point mutations are listed here; primer sets for the remaining, nonessential point mutations can be obtained upon request. All constructs were confirmed via sequencing (Genewiz, Inc.).

Transfections. Transient transfections were carried out in 24-well plates (2×10^5 cells per well) via Lipofectamine treatment (Invitrogen) as previously described (33). Unless otherwise indicated, transfection amounts per well were as follows: 500 ng luciferase reporter, 50 ng AP-1 plasmid, 50 ng STAT3 plasmid, 5 ng *Renilla* plasmid. Twenty-four hours after transfection, cells were treated with IL-6/IL-6 soluble receptor (or TPA or OSM) for 6 h. Luciferase assays were performed using the Dual Luciferase assay kit (Promega). Results shown in the figures are normalized against the internal control, *Renilla* luciferase activity. Each transfection condition was performed in triplicate in a given experiment.

siRNA experiments. Small interfering RNA (siRNA) was generated using a Recombinant Dicer enzyme kit from Gene Therapy Systems (GTS) and a Silencer siRNA construction kit (Ambion). Briefly, a double-stranded DNA template consisting of the first 200 nucleotides of the Jun or Fos gene was created, flanked with a short T7 promoter. Double-stranded RNA was made and purified from these templates using the Ambion kit. siRNA was generated from double-stranded Jun or Fos RNA with recombinant Dicer enzyme from GTS and purified using the GTS kit. H35 cells were transfected with 100 nM siRNA (or as indicated in Fig. 1D), via a Lipofectamine protocol. Treatment with siRNA (or mock treatment) was carried out over 48 or 72 h, and then cells were stimulated (or not) with IL-6/receptor and Dex. Total protein was collected in whole-cell lysis buffer (see "Protein extraction and purification"), and total mRNA was extracted with TRIzol (Invitrogen protocol). Western blot analysis and reverse transcription-PCR (RT-PCR) were carried out by standard methods (4).

GST pulldown experiments. GST-STAT3 truncations were expressed and purified from *Escherichia coli* as previously described (33). Radiolabeled Jun and Fos truncated proteins were generated using the TNT-T7 Quick-Coupled transcription/translation kit (Promega). Briefly, Jun and Fos truncations were made via PCR using the following primers (T7 promoter inserted at beginning of sense strands for TNT-T7-coupled reaction): c-Jun(253-315), sense, 5′-GCGTAATA CGACTCACTATAGGGAGAATGAAGGCGGAGAGGAAGCGC-3′; antisense, 5′-GTGGTTCATGACTTTCTGTTTAAGCTG-3′; c-Jun(105-253), sense, 5′-GCGTAATACGACTCACTATAGGGAGAATGGATGAGCAGGAGGGGT TCG-3′; antisense, 5′-CCGCTCCTGGGACTCCATGTCGAT-3′; c-Jun(253-293), sense, 5′-GCGTAATACGACTCACTATAGGGAGAATGAAGGCGGAGAGG AAGCGC-3′; antisense, 5′-CTCCGAGTTCTGAGCTTTCAAGGT-3′; c-Jun(265-315), sense, 5′-GCGTAATACGACTCACTATAGGGAGAATGGCTGCCTCCA AGTGCCGA; antisense, 5′-GTGGTTCATGACTTTCTGTTTAAGCTG-3′; c-Fos(1-138), sense, 5′-GCGTAATACGACTCACTATAGGGAGAATGATGTTC TCGGGCTTCAACG-3′; antisense, 5′-CTCTTCTTCTTCTGGAGATAACTG; c-Fos(139-380), sense 5′-GCGTAATACGACTCACTATAGGGAGAATGAAAA GGAGAATCCGAAGGG-3′; antisense, 5′-TCACAGGGCCAGCAGCGTGG GT-3′; c-Fos(139-200), sense, 5′-GCGTAATACGACTCACTATAGGGAGAAT GAAAAGGAGAATCCGAAGGG-3′; antisense, 5′-GTGAGCTGCCAGGATG AACTCTAG. For in vitro translation of Jun and Fos truncations, PCR products (above) were used in program-coupled transcription/translation reactions in the presence of [^{35}S]methionine (Dupont/NEN) according to a Promega protocol. GST-protein binding assays with translation products were conducted as previously described (33). Final products were eluted in sodium dodecyl sulfate (SDS) gel-loading buffer and resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE).

Protein extraction and purification. Purified phosphorylated STAT3 protein was prepared as previously published (23a). Total cell, cytosolic, and nuclear extracts from H35 and HepG2 cells were prepared as previously described (26).

Purification of Myc-GST-tagged proteins was conducted as follows. 293 cells were transfected with GST-Jun and Fos plasmids via Lipofectamine for 24 h in 150-mm plates. Cells were similarly harvested in 1× cold phosphate-buffered saline (PBS). Cell pellets were resuspended in GST-binding buffer (1× PBS, pH 7.4, 1 mM EDTA, and fresh dithiothreitol [0.5 mM], fresh phenylmethylsulfonyl fluoride [1 mM], and fresh protease inhibitor cocktail [100×; Calbiochem]). Cells were lysed via two freeze-thaw cycles in liquid N_2 and a 37°C water bath. Lysates were then passed five times through an 18-gauge needle and spun down for 15 min at 14,000 rpm at 4°C. Supernatant was passed through a 0.45-μm filter and incubated with GST-Sepharose beads (prewashed twice with GST-binding buffer) with rocking overnight at 4°C. GST fusions were obtained via column purification (Poly-prep chromatography columns; Bio-Rad). Beads were washed three times with GST-binding buffer, and proteins were eluted in GST elution buffer (50 mM Tris, pH 8.0, 10 mM glutathione). Purification of eluted proteins was analyzed via Western blotting (myc antibody).

EMSA. A ^{32}P-labeled oligonucleotide presenting strong AP-1 and STAT sites was generated as previously described (31) (sequence: 5′-CGCTTGA**TGACTC AGCCGGAATCAT*TTCCCGTAA***ATCAT-3′ [bolface, strong AP-1 binding sequence; boldface italics, strong STAT binding sequence]). GST-purified Jun proteins (wild type and mutant) were produced (see above) and used in electrophoretic mobility shift assays (EMSA), with purified phosphorylated STAT3. EMSA analysis was conducted as previously described (31). Samples were run on 4% native acrylamide gels, dried, and exposed to film.

RESULTS

Removal of AP-1 proteins via siRNA treatment results in down-regulation of IL-6-inducible STAT3-dependent genes. The rat α2-M gene is an acute-phase response gene, transcription of which in H35 rat hepatoma cells is stimulated by IL-6 through STAT3 and is boosted considerably by simultaneous dexamethasone treatment (2, 10, 12, 14, 21). Both the presence and orientation of the AP-1 site in the α2-M promoter were necessary for maximal induction of the α2-M promoter in H35 cells transfected with reporter constructs (17). Furthermore, chromatin precipitation experiments showed that c-Jun and also apparently c-Fos were present on the endogenous α2-M promoter prior to induction (17). To determine the importance of AP-1 factors in cooperating in STAT3 activation of this promoter, we used siRNA knockdown experiments directed against either c-Jun or c-Fos. Cells treated with c-Jun or c-Fos siRNA for 48 h, compared to untreated or mock-treated controls, showed a decrease in both c-Jun and c-Fos mRNA

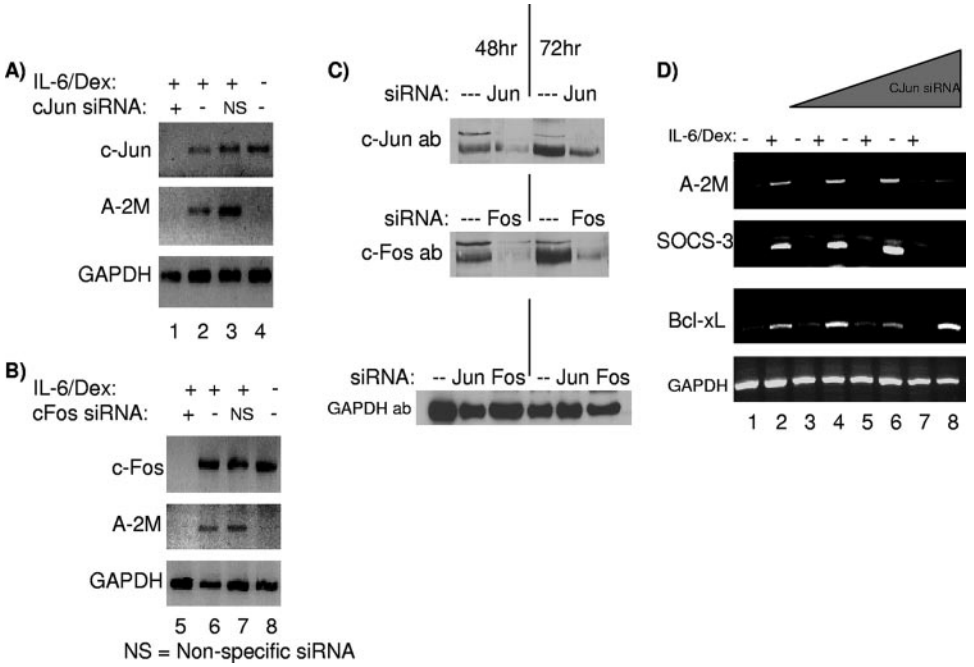

FIG. 1. Down-regulation of STAT3-dependent genes after AP-1 siRNA treatment. (A) RT-PCR assay for c-Jun and α2-M mRNA expression. H35 cells were treated with 100 nM c-Jun siRNA (lane1) or nonspecific siRNA (NS; lane 3) for 48 h or were untreated (lanes 2 and 4) and then stimulated (lanes 1 to 3) with IL-6/Dex for 2 h or not stimulated (lane 4). Total RNA was extracted, reverse transcribed, and analyzed via PCR. GAPDH was used as an internal control. (B) RT-PCR assay for c-Fos and α2-M expression. H35 cells were treated with 100 nM c-Fos siRNA (lane 5) or nonspecific siRNA (lane 7) for 48 h or untreated (lanes 6 and 8) and then stimulated (lanes 5 to 7) with IL-6/Dex for 2 h or not stimulated (lane 8). Total RNA was extracted, reverse transcribed, and analyzed via PCR. GAPDH was used as an internal control. (C) Western blot for c-Jun or c-Fos protein. H35 cells were treated with 100 nM siRNA directed against c-Jun or c-Fos for 48 or 72 h or not treated. Protein extracts were resolved by 10% SDS-PAGE and blotted with antibodies (ab) for c-Jun (top) or c-Fos (middle). GAPDH was used as an internal control (bottom). (D) RT-PCR assay for α2-M, SOCS-3, and Bcl-xL expression. H35 cells were treated with increasing concentrations of c-Jun siRNA (lanes 3 and 4, 25 nM; lanes 5 and 6, 50 nM; lanes 7 and 8, 100 nM) for 48 h and then stimulated (lanes 2, 4, 6, and 8) with IL-6/Dex for 2 h or not stimulated (lanes 1, 3, 5, and 7). Total RNA was extracted, reverse transcribed, and analyzed by PCR. GAPDH was used as an internal control.

levels and c-Jun and c-Fos protein (Fig. 1A to C). Control or siRNA-treated cells were tested for α2-M mRNA increase in response to IL-6/Dex; a strong suppression of the normal increase in α2-M mRNA was observed in cells treated specifically with either c-Jun (Fig. 1A) or c-Fos (Fig. 1B) siRNA. To determine if other IL-6-inducible genes were similarly affected by diminished AP-1 protein levels, we measured the expression of two other IL-6-inducible genes, the SOCS-3 (suppressor of cytokine signaling 3) and BclxL genes, after siRNA treatment. The IL-6-dependent induction of the SOCS-3 gene was also suppressed by siRNA treatment, while the BclxL gene was not. (Likewise, survivin mRNA was induced by IL-6 but was not suppressed by c-Jun or c-Fos siRNA treatment [data not shown; Fig. 1D]). In contrast, c-Fos siRNA treatment had no effect on the induction of SOCS-3 mRNA (data not shown).

Identification of STAT3-binding domain in c-Jun and c-Fos proteins. Although there are noteworthy differences in both structure and function of c-Jun and c-Fos, the two proteins do possess several homologous domains, the most significant of which are their B-zip DNA-binding domains (Fig. 2A). We previously found that a fragment of STAT3 (107 to 377) bound to a portion of c-Jun (amino acids 105 to 334) (33). To more precisely define the binding region, truncated c-Jun mutants were prepared and tested with GST-STAT3 fusion proteins in a pulldown analysis. Only a small region of the c-Jun carboxy terminus, consisting primarily of the B-zip domain (amino acids 253 to 315), was sufficient for in vitro interaction (Fig. 2B, top panel, lane N). The association between these two proteins was lost if the B-zip domain of c-Jun was further truncated (Fig. 2B, bottom two panels, lane N). As expected from previous results, none of the c-Jun truncations bound to the STAT3 C-terminal portion of the STAT3 DNA binding domain (377 to 770) (Fig. 2B, lane C). To further demonstrate the specificity with which these two protein segments interact, we incubated the c-Jun truncations with a mutated STAT3 protein (STAT3-TKR) previously shown not to interact with a larger segment of c-Jun (33). The c-Jun B-zip domain truncation also failed to interact with the STAT-TKR mutant (Fig. 2C, lane T).

We have found no report of c-Fos interacting directly with STAT3. Since c-Fos siRNA blocked the IL-6/Dex induction of α2-M mRNA in H35 cells, we prepared c-Fos truncations and tested for interaction with the GST-STAT3 segment that interacts with c-Jun. Indeed c-Fos did associate in vitro with the same domain of STAT3 as c-Jun, and c-Fos binding was also mediated by the B-zip domain of c-Fos (Fig. 2D, bottom two panels, lane N). Neither the amino terminus (amino acids 1 to 138; Fig. 2D, top panel, lane N) nor the extreme carboxyl terminus of c-Fos (amino acids 200 to 380; data not shown) was capable of interacting with this STAT3 segment. Finally, the

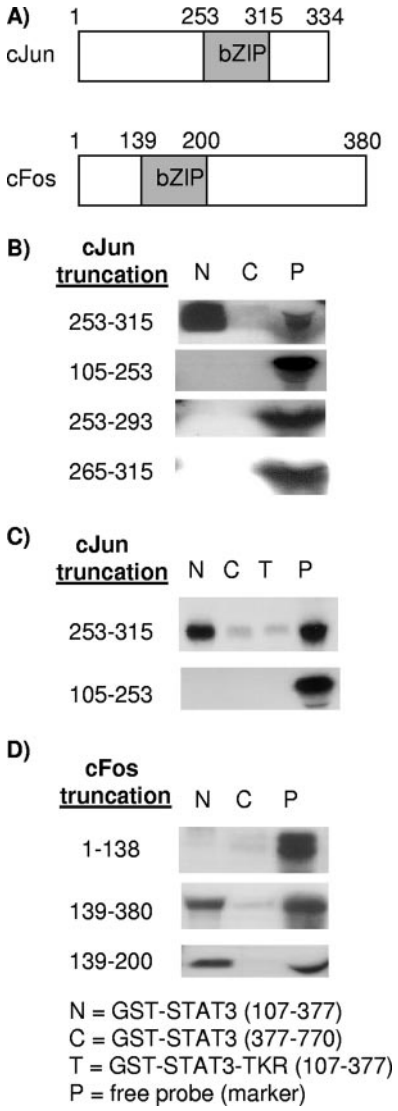

FIG. 2. The B-zip domain of AP-1 mediates binding to STAT3. (A) Schematic diagram of AP-1 proteins c-Jun and c-Fos. (B to D) GST pulldown of AP-1 by STAT3. GST-STAT3 fusions were incubated with radiolabeled c-Jun or c-Fos truncations and resolved by 10% SDS-PAGE (amino acid regions tested are shown). (B) The B-zip domain of c-Jun is necessary and sufficient for binding to STAT3. (C) The B-Zip domain of c-Jun is unable to bind to the STAT3-TKR mutant. (D) The B-zip domain of c-Fos (homologous to c-Jun) is necessary and sufficient for binding to STAT3.

FIG. 3. A single amino acid in AP-1 is essential for STAT3 binding in vitro. GST pulldown of AP-1 by STAT3 is shown. GST-STAT3 fusions were incubated with radiolabeled c-Jun or c-Fos truncations and resolved by 10% SDS-PAGE (AP-1 B-zip domain mutations tested are shown). (A) Quadruple mutation in the B-zip domain of c-Jun disrupts binding to STAT3. (B) Single mutation in the B-zip domain of c-Jun (arginine 261) disrupts binding to STAT3. (C) Single mutation of homologous arginine in the B-zip domain of c-Fos (arginine 146) disrupts binding to STAT3.

carboxyl portion of STAT3 (377 to 770) was incapable of associating with all c-Fos truncations (Fig. 2D, lane C). Collectively, these data suggest that the residues responsible for binding to STAT3 reside either within or immediately proximal to the B-zip/DNA-binding domain of either c-Jun or c-Fos.

A single amino acid in either c-Jun or c-Fos is required for in vitro STAT3 binding. The association of c-Jun with N-FAT while bound to DNA involves residues near to but not within the DNA binding region of c-Jun or c-Fos (7). Using those results as a clue, we mutated c-Jun and c-Fos residues in similar regions and tested for interaction of STAT3 with a GST-tagged STAT3 segment (residues 107 to 377). Most of the first group of c-Jun mutants still bound to STAT3 (Fig. 3A), but a quadruple mutation of residues 256, 257, 260, and 261 prevented STAT3 from interacting with c-Jun (Fig. 3A, bottom panel, lane N). We then found that a single point mutation in R261 (to either alanine [A] or the negatively charged aspartic acid [D]) was sufficient to inhibit the STAT3/c-Jun interaction (Fig. 3B). As the B-zip domains of c-Jun and c-Fos are quite similar, we mutated the homologous arginine in c-Fos (R146A), which significantly reduced the ability of the c-Fos B-zip domain to interact with STAT3 (Fig. 3C, bottom panel, lane N). Likewise, mutation R146D also disrupted c-Fos association with STAT3 (data not shown). Taken together, these results reveal that the B-zip regions of both c-Jun and

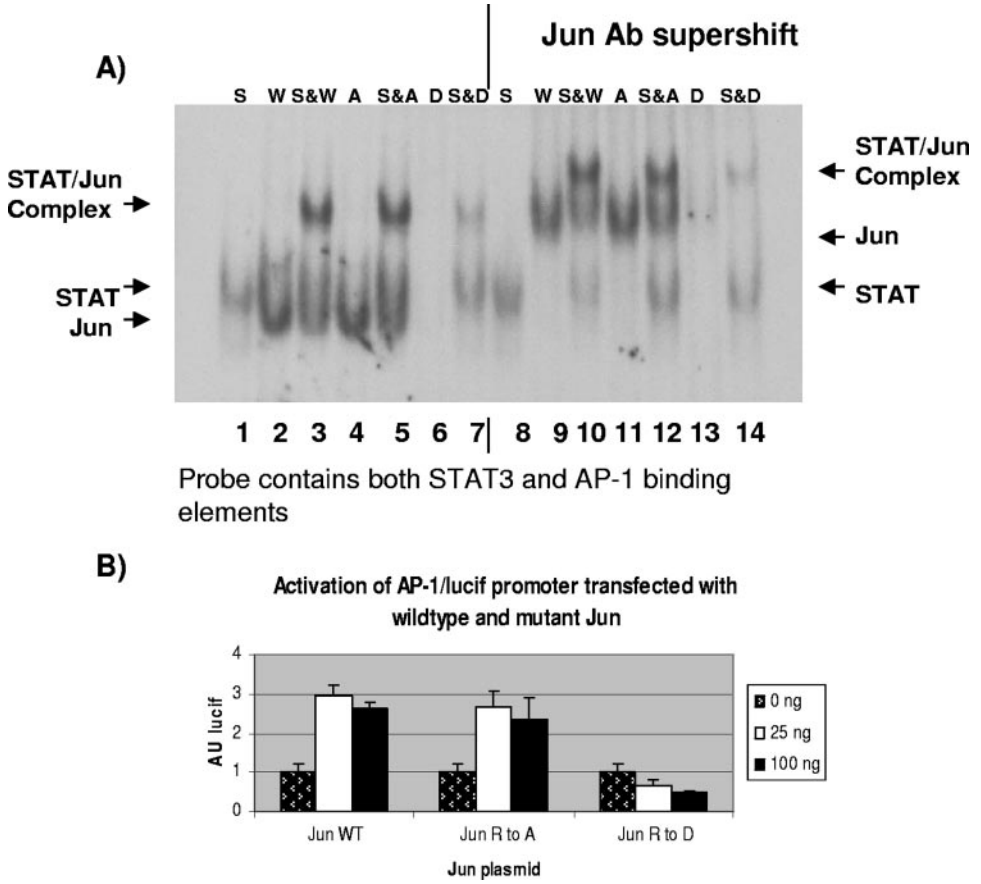

FIG. 4. STAT3 and c-Jun form a tertiary complex with DNA. (A) EMSA of purified phosphorylated STAT3 and/or purified Jun proteins with a ^{32}P-labeled oligonucleotide, presenting strong AP-1 (boldface) and strong STAT (italic boldface) binding sequences (5′-CGCTTGA**TGACTC A**GCCGGAATCAT***TTCCCGTAA***ATCAT-3′). Phospho-STAT3 (lanes 1 and 8), wild-type Jun (lanes 2 and 9), and R261A Jun (lanes 4 and 11) all produced significant gel shifts with the probe. No shift was observed with R261D Jun (lanes 6 and 13). A tertiary complex of phospho-STAT3/Jun/DNA formed in the presence of either wild-type Jun (lanes 3 and 10) or R261A Jun (lanes 5 and 12). The stability of this complex was significantly lower, however, in the presence of R261D Jun (lanes 7 and 14). The presence of Jun (lanes 8 to 14) and STAT3 (data not shown) in shifted complexes was confirmed by supershift with a specific antibody. (B) Luciferase (lucif) activity of an AP-1 promoter-luciferase construct was measured, following overnight transfection with various Jun plasmids (as indicated), in HepG2 cells (stimulation for 6 h with TPA). Two concentrations of each plasmid were measured (25 and 50 ng), and luciferase expression was normalized against cells not transfected with Jun. In summary, both wild-type and R261A mutant Jun proteins are transcriptionally active, while R261D Jun is unable to active transcription.

c-Fos are important for the interaction with STAT3 and that R261 in c-Jun (or R146 in c-Fos) is particularly important.

c-Jun mutations and cooperative DNA binding. Both STAT3 and c-Jun are, of course, known to bind DNA. Further, it has been shown that phospho-STAT3 and c-Jun together will bind jointly to DNA containing both STAT3 and c-Jun binding sites (31). We determined by EMSA the effects of the c-Jun mutations discussed above on the binding to labeled DNA with both STAT and c-Jun sites of purified c-Jun and phospho-STAT3 alone and together. Wild-type c-Jun as well as the R261A and the R261D mutant c-Jun proteins were examined. As expected, either phosphorylated STAT3 or wild-type c-Jun alone bound and gave single bands (Fig. 4A; lanes 1 and 2). The R261A c-Jun mutant also bound DNA (lane 4); however, the R261D protein by itself did not bind DNA (lane 6). In this protein the negatively charged aspartic acid is very close to the amino acids known from crystallography to be responsible for c-Jun/DNA contacts (7, 11).

When the two wild-type proteins (lane 3) or wild-type phospho-STAT3 and R261A c-Jun (lane 5) were added together, a slower-migrating complex that contained both STAT3 and c-Jun proteins, as demonstrated by shifting this slower-moving band with c-Jun antibody (lanes 10 and 12), was observed; the slower-moving complex could also be shifted by treatment with STAT3 antibody (data not shown). When the R261D c-Jun mutant, which by itself did not bind DNA, was added with STAT3, a smaller amount of the slowly migrating band was observed, indicating a cooperative but weaker interaction between STAT3, the R261D protein, and DNA (lane 7). This weak band was also supershifted with the anti-c-Jun antibody (lane 14) or anti-STAT3 antibody (data not shown). As a final test of the c-Jun mutants we performed transfection analysis with a reporter construct containing only AP-1 binding sites. The wild-type and R261A mutant (both of which bind DNA) gave approximately equal stimulation of transcription of the plasmid, while the R261D mutant, which fails to bind DNA on its own, gave no stimulation (Fig. 4B) and, in fact, reduced the signal due to endogenous protein.

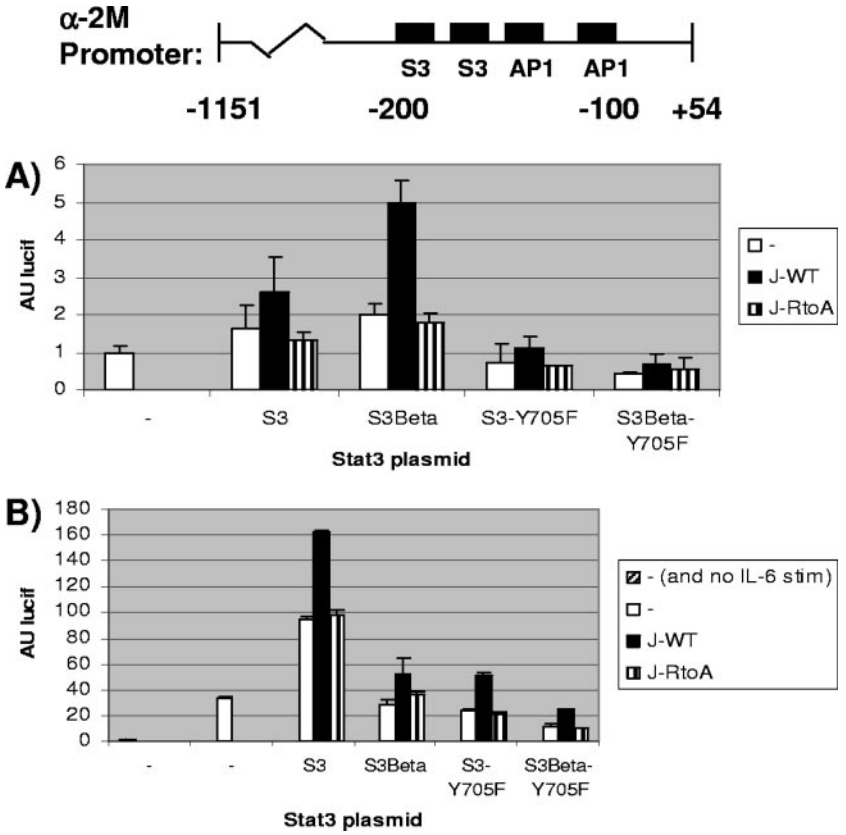

FIG. 5. STAT3 and AP-1 cooperation is dependent upon tyrosine phosphorylation of full-length STAT3. Luciferase activity of the −1151/+54 α2-M promoter-luciferase construct was measured, following overnight transfection with wild-type and mutant STAT3, STAT3β, and AP-1 plasmids (as indicated) in HepG2 cells (stimulated for 6 h with IL-6 or unstimulated). (A) In unstimulated cells, transfection with STAT3B increases transcription of the α2-M promoter, which is further augmented via c-Jun transfection. Mutation of tyrosine 705 abolishes this augmentation. (B) In cells stimulated with IL-6, STAT3 transfection significantly increases induction of the α2-M promoter, which is further augmented by c-Jun. Again, mutation of T705 abrogates the synergism between c-Jun and STAT3. S3, STAT3; S3Beta, STAT3β; S3-Y705F, STAT3 tyrosine 705 mutant; S3Beta-Y705F, STAT3β tyrosine 705 mutant; J-WT, wild-type Jun; J-RtoA, mutant Jun.

The original report of transcriptional cooperation between c-Jun and STAT3 utilized STAT3β with no activating ligand to purposely cause STAT3 tyrosine phosphorylation (24). We tested a similar system to determine if, in fact, the Y705 residue was required for c-Jun/STAT3 cooperation during a transfection assay (Fig. 5). The vectors used contain a segment of the α2-M promoter that has two STAT and two c-Jun binding sites. The cells used, HepG2, contain endogenous levels of c-Jun and STAT3 and therefore give a basal level of transcription of vectors with c-Jun and STAT3 binding sites. First, there was in untreated cells a small increase upon transfection of STAT3β alone, which was abolished by STAT3β Y705F (Fig. 5A). Again, with no IL-6 stimulation there was an increase in signal due to the addition of c-Jun and wild-type full-length STAT3 but a much greater increase due to the addition of c-Jun and STAT3β. (These increases were suppressed by the c-Jun R261A mutant; this use of this mutant will be further analyzed below [see Fig. 6].) Most important, when STAT3β Y705F was used, there was suppression in every case. We interpret these results to mean that the well-described low level of spontaneous, non-ligand-dependent phosphorylation of STAT3 and particularly of STAT3β is the basis for these results: the positive interaction of c-Jun and STAT3β closely depends on the presence of Y705. This effect is even more apparent in cells transfected as above but treated with IL-6 to stimulate STAT tyrosine phosphorylation (Fig. 5B). Once again STAT3 alone gives a boost to the background level but STAT3β Y705F exerts a strong negative effect, reducing the background threefold. Wild-type STAT3 plus c-Jun give the strongest cooperative signal, and STAT3β Y705F reduces that level by sevenfold. These results clearly indicate that c-Jun/STAT3 cooperation is between tyrosine-phosphorylated full-length STAT3 and c-Jun.

c-Jun mutations affect the response of the α2-M promoter. Using this system, we determined whether the cooperative interaction between wild-type c-Jun and STAT3 to drive transcription would be affected by the c-Jun mutants that break up the in vitro c-Jun/STAT3 interaction. As noted before (33), STAT3 alone, but not c-Jun alone, boosted the background response to IL-6 about twofold (Fig. 6A). The addition of both wild-type STAT3 and wild-type c-Jun boosted the IL-6-induced response a further twofold, implying cooperation between STAT3 and c-Jun.

We next determined in several experiments the effect of mutations in c-Jun on the cooperation in driving transcription. Figure 6B shows one such experiment in which the cooperation

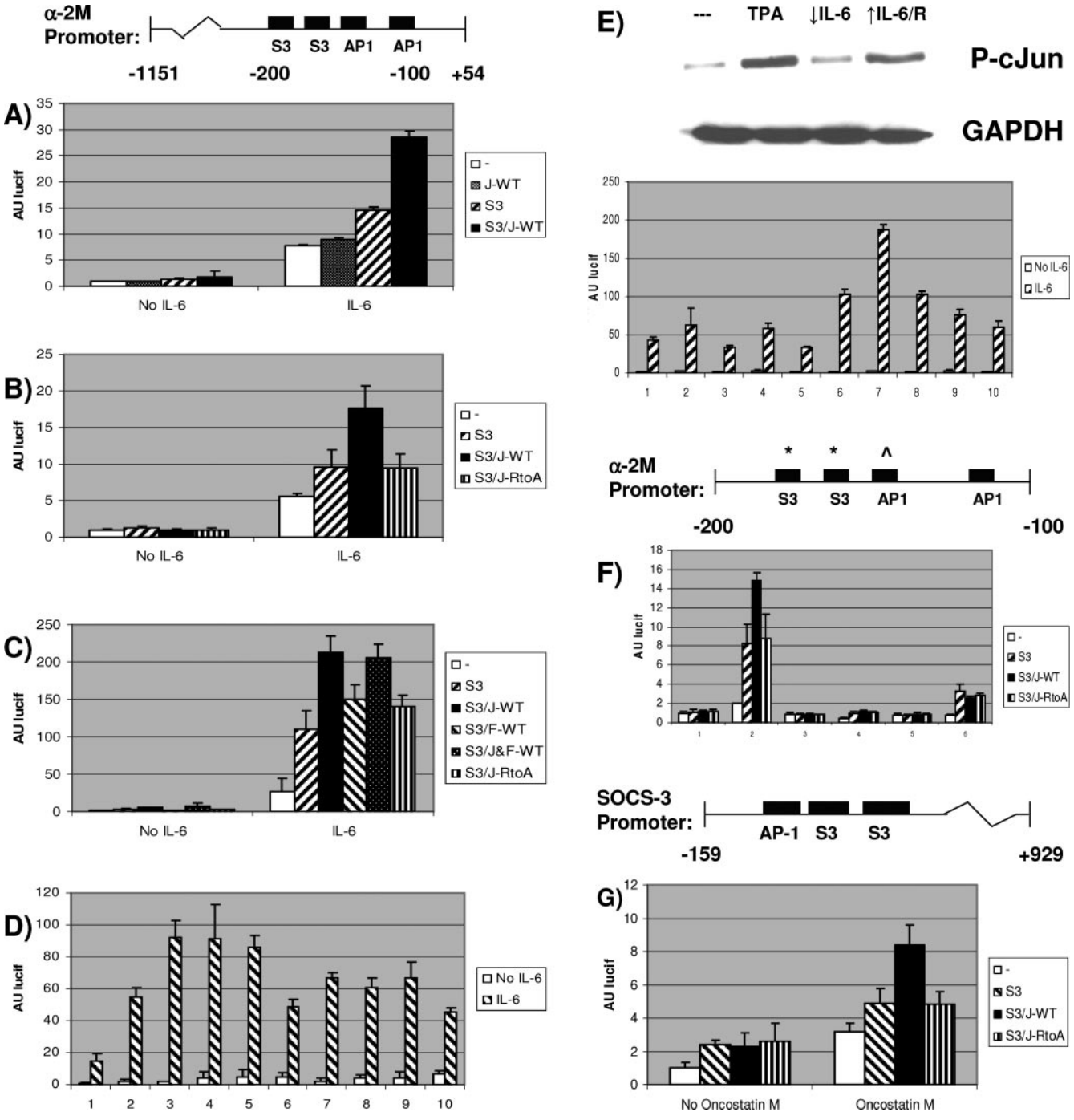

FIG. 6. STAT3 and AP-1 mediate transcriptional activity of α2-M and SOCS-3 luciferase constructs. Luciferase activity of the −1151/+54 α2-M promoter-luciferase construct (wild type; A through E) or the −200/−100 α2-M promoter-luciferase constructs (wild type and mutant; F) was measured, following overnight transfection with STAT3 and/or various AP-1 plasmids (as indicated) in HepG2 cells (stimulated for 6 h with IL-6 or unstimulated). Additionally, luciferase activity of the −159/+929 SOCS-3 promoter-luciferase construct (wild type; G) was measured, following overnight transfection with STAT3 and/or various AP-1 plasmids (as indicated) in HepG2 cells (stimulated for 6 h with OSM or unstimulated). (A) Transfection with STAT3 induces transcription of the α2-M promoter, which is further augmented via c-Jun transfection. (B) Mutation of arginine 261 in c-Jun prevents synergistic activation of the α2-M promoter by STAT3 and c-Jun. (C and D) Transfection of various combinations of wild-type and mutant c-Jun/c-Fos plasmids demonstrates that cooperative activation of the α2-M promoter requires both STAT3 and wild-type c-Jun. (D) Lane 1, no transfection; lane 2, STAT3 (S3); lane 3, S3/wild-type Jun (J-WT); lane 4, S3/J-WT/wild-type c-Fos (F-WT); lane 5, S3/J-WT/R261A c-Fos; lane 6, S3/R261A c-Jun; lane 7, S3/F-WT; lane 8, S3/R146A c-Fos; lane 9, S3/R146A c-Jun/F-WT; lane 10, S3/R261A c-Jun/R261A c-Fos. (E, top) In HepG2 cells high levels of IL-6 plus soluble IL-6 receptor results in phosphorylation of c-Jun. (E, bottom) Mutation of serine 63 and 73 in c-Jun prevents cooperative activation of the α2-M promoter by STAT3 and c-Jun during transfection of HepG2 cells as in panels A to D. Lane 1, no transfection; lane 2, J-WT; lane 3, mutant Jun R261A (J-RtoA); lane 4, mutant Jun S63A (J-StoA); lane 5, J-RtoA, J-StoA; lane 6, S3; lane 7, S3/J-WT; lane 8, S3/J-RtoA; lane 9, S3/J-StoA; lane 10, S3/J-RtoA, J-StoA. (F) Presence of both STAT3 and AP-1 elements in the α2-M promoter is necessary for transcriptional activation to occur. *, mutated STAT3 sites in α2-M S3 mutant promoter;

between wild-type c-Jun and STAT3 is blocked essentially completely by the R261A mutant c-Jun protein. This result is repeated in Fig. 6C and D, where potential effects of mutations in c-Fos were also examined. The summary conclusion of these results is that, while c-Jun R261A or R261D consistently decreased the STAT3/c-Jun cooperation in transcription, there was, in this assay, no effect of c-Fos on cooperation with STAT3, nor was there any effect of mutant c-Fos protein.

Stimulation of transcription by c-Jun has long been known to require phosphorylation on residues in the N terminus, notably S63 and S73 (15, 27). One important role of this phosphorylation is removal of the inhibitor histone deacetylase 3 (29). Moreover IL-6 has been reported to activate some mitogen-activated protein kinase pathways capable of phosphorylating c-Jun in certain cell types (13, 22). We found that c-Jun S63,S73 phosphorylation occurs in HepG2 cells in response to IL-6 plus the soluble IL-6 receptor in a dose-dependent manner (Fig. 6E, top). We therefore determined whether c-Jun/STAT3 transcriptional cooperation required the S63 and S73 residues of c-Jun. Following transfection and IL-6 stimulation (Fig. 6E, bottom) the wild-type c-Jun boosted STAT3 transcription as usual about twofold (lane 7). The S63A/S73A c-Jun mutant protein suppressed this cooperation even more than the R261A c-Jun mutant (lane 8), and use of both mutants (R261A plus S63/S73A, lane 10) together resulted in even greater suppression. Thus, the STAT3/c-Jun cooperation on the α2-M promoter definitely depends on a wild-type c-Jun phosphorylated on S63/S73.

There are two STAT sites and two AP-1 sites in the 200 nucleotides upstream from the RNA start site in the α2-M promoter. Mutation of the STAT3 sites or of the AP-1 site closest to the STAT3 sites renders promoter constructs non-inducible (Fig. 6F), while mutation of the more distal AP-1 site reduces responsiveness by only about 25% (17). So the cooperation monitored in the experiments of Fig. 6A to E is likely due to interactions between STAT3 and c-Jun at the AP-1 site closest to the STAT site. Earlier experiments showed that a 5-bp insertion separating the contiguous STAT site and AP-1 site decreased a transcriptional response (17).

To examine whether other promoters showed STAT3/c-Jun cooperation, we examined a reporter plasmid containing the SOCS-3 promoter region transfected in HepG2 cells (Fig. 6G) (3). In the promoter the previously identified STAT3 and c-Jun sites lie close together, about 100 bp upstream of the RNA start site. The response of this promoter to supplemental STAT3 and wild-type c-Jun after OSM stimulation (OSM also acts through IL-6 activation of STAT3) was about twofold. The stimulation was reduced considerably when either c-Jun R261A or R261D was used instead of wild-type c-Jun.

DISCUSSION

Cooperation between transcription factors bound to neighboring regions, most often closely spaced, in promoter DNA is extremely common in the activation of mammalian genes (6). In a few cases not only have specific proteins been identified but also, by crystallography, structural contacts between proteins bound to DNA containing the multiple binding sites have been identified (7, 23).

Persistently active STAT3 has been identified in many human cancers and appears to be required for continued growth or resistance to apoptosis in cultured human cancer cell lines (32). Therefore, interaction of STAT3 with other proteins in driving transcription commands considerable interest. If such interactions can be localized (preferably by crystallography) and shown to be important in transcription, it is possible that antioncogenic targets for small-molecule drugs will be uncovered.

We have followed this logic for STAT3 and c-Jun because the c-Jun protein was the first nuclear oncoprotein to be discovered and has been demonstrated to be involved in cell transformation by many oncogenes (28). Therefore we were particularly interested in studying the STAT3/c-Jun interaction. We earlier found that a segment of STAT3 containing the coiled-coil and a portion of the DNA binding domain of STAT3 would interact with the COOH-terminal half of c-Jun (28). This interaction was interrupted by mutation of residues both in the coiled-coil and residues (T346A, K348A, and R350A) in a portion of the DNA binding domain away from the DNA contact region.

We now show that a limited portion of the c-Jun protein, the β-zip domain, which contains the DNA binding domain, has the STAT3-interacting region. Moreover, there is a single crucial residue, R261, in c-Jun mutations that blocks the STAT3 interaction. The c-Fos protein, which is similar to c-Jun in structure and frequently forms heterodimers with c-Jun, also interacts similarly with STAT3. c-Fos also possesses a single residue, R146, which when mutant interrupts STAT3 interaction. Removal of either c-Jun or c-Fos by siRNA in H35 rat hepatoma cells reduced STAT3-dependent induction of α2-M, and removal of c-Jun, but not c-Fos, also reduced SOCS-3 expression. We were able to show in transient transfections with c-Jun but not c-Fos that c-Jun and STAT3 cooperate in driving transcription of α2-M and SOCS-3 reporter constructs. This cooperation depends on tyrosine residue 705 in STAT3. Early results (24) suggesting that STAT3β without ligand stimulation could cooperate with STAT3 were shown likely be a result of low-level tyrosine phosphorylation due to factors in serum-grown cells. Finally and most significantly, the mutations in c-Jun that disrupt the in vitro interaction of STAT3 and c-Jun also decreased greatly the transcriptional coopera-

^, mutated AP-1 site in α2-M AP-1mutant promoter. Lanes 1 and 2, wild-type α2-M promoter; lanes 3 and 4, S3 mutant α2-M promoter; lanes 5 and 6, AP-1 mutant α2-M promoter; lanes 2, 4, and 6, cells stimulated with IL-6. (G) Transfection with STAT3 induces transcription of the SOCS-3 promoter, which is further augmented via c-Jun transfection. Mutation of arginine 261 to alanine or aspartic acid prevents synergistic activation of the promoter by STAT3 and c-Jun. c-Jun is incapable of stimulating induction above background levels in the absence of exogenous STAT3.

tion on two STAT3-dependent promoters, as did removing the positive-acting phosphorylation sites S63 and S73 in c-Jun. Searches through the genomic sequence reveal a number of genes that have closely spaced candidate STAT3 and AP-1 sites. This fact plus the present results portend that the demonstrable interaction between the coiled-coil domain of STAT3 (where the TKR mutations lie) and basic region of c-Jun harboring R261 will be a common event.

We have attempted to dock the known structure of phosphorylated STAT3 bound to DNA and c-Jun bound to DNA using the α2-M binding sites to compare to the published N-FAT/c-Jun/DNA structure. It appears that no contact between the bound proteins would occur without a bend in the DNA or, as is unlikely, a change in protein structure. We believe that the present results elevate the described interaction of STAT3 and c-Jun to the status of a useful target to search for compounds that would interrupt STAT3-dependent gene activation and that could conceivably be anticancer leads.

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