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Interactions between STAT and Non-STAT Proteins in the Interferon-Stimulated Gene Factor 3 Transcription Complex

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The first STAT-containing transcription factor to be studied, the alpha-interferon-induced ISGF3, is composed of a Stat1:2 heterodimer and a weak DNA-binding protein, p48, that is a member of a growing family of proteins similar to the so-called interferon regulatory factor (IRF-1). The p48 and Stat1:2 heterodimer do not associate stably in the absence of DNA, but we show that amino acids ~150 to 250 of Stat1 and a COOH-terminal portion of p48 exhibit physical interaction, implying contact that stabilizes ISGF3. Moreover, amino acid exchanges within the Stat1 contact region diminish or abolish the functional activity of Stat1. This protein interaction domain may be important in other STAT proteins to recruit partners to multiprotein transcription factors.

Much attention in the eukaryotic transcription field has focused recently on the very large number of proteins that assist in the initiation of transcription by RNA polymerase II (pol II) (25). Many of these proteins which are present in all cells and are part of the initiation complex are described as general transcription factors and may be involved in every initiation event. Other of these proteins such as the coactivators and TAFs (proteins associated with the TATA box-binding protein) interact both with general transcription factors and with a third group of required transcription factor proteins that specifically bind at DNA sites distant from the initiation site (promoter and enhancer sites). The proteins in this third group have often been thought of as "regulatory" proteins, because they are either not present in all cells or not active at all times. Finally, DNA binding sites within the promoter and enhancer regions also have been recently recognized to be a complex mixture of sites for common and regulatory proteins. For example in the beta-interferon (IFN-β) enhancer, to achieve maximal induced transcription, at least six polypeptides, only one or two of which exhibit induced behavior, must all bind and make required contacts with each other and with the DNA in a 60-bp stretch (23, 43). Thus, the protein-protein interactions of regulatory transcription factors bound to their specific binding sites can be expected to be crucial in many if not most cases of induced transcription in eukaryotic cells.

The STATs are a recently recognized class of transcriptional regulatory proteins that serve the dual function of signal transduction and transcriptional activation (6, 36). The STATs mediate the activation of immediate-response genes as part of a polypeptide-induced signal transduction system known as the JAK-STAT pathway. The STAT molecule binds to phosphotyrosine residues in the cytoplasmic domain of a transmembrane receptor that has been activated by its specific polypeptide ligand (10, 13, 41, 42); the STAT then becomes phosphorylated on tyrosine and dimerizes via intermolecular SH2-phosphotyrosine interactions. The dimeric factor is then translocated to the nucleus, where it binds specific DNA sites and drives transcription.

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The initial identification of STAT proteins as transcription factors came through purification from IFN-α-treated cells of a complex, three-protein factor, interferon-stimulated gene factor 3 (ISGF3). ISGF3 was assembled after IFN treatment from preexisting cytoplasmic proteins. This regulatory factor binds to a 15-bp DNA element, the ISRE (interferon-stimulated response element) (8) found in the enhancers of IFN-α-responsive genes. When the genes encoding the three ISGF3 proteins were cloned, it was found that two large proteins, 91 and 113 kDa (9, 37), were joined on the DNA by a third, smaller molecule, p48. The two larger proteins are now known as Stat1 and -2, and represent the first members defining the STAT family. The smaller protein, a weak ISRE-binding protein on its own (19), was not a STAT but belonged to another family of DNA-binding proteins (45) now known as the interferon regulatory factor (IRF) family (26). Although not a STAT, p48 was essential for the formation of ISGF3. Stat1 and Stat2 are phosphorylated on tyrosine and form a stable heterodimer in response to IFN-α (31, 38), but early experiments showed no association of the Stat1:2 heterodimer with p48 in the absence of DNA (19, 31). Since UV cross-linking experiments demonstrated that the p48 and Stat1:2 heterodimer are all bound to the ISRE within a few base pairs to form the stable ISGF3 complex (31) and p48 by itself is only a weak DNA-binding protein, physical contact between the STATs and p48 seemed likely. In the present work we used somatic-cell genetics (29), yeast interaction trapping (11), and in vitro biochemical tests of protein-protein association to provide the first evidence for and identification of the residues involved in the physical contact between Stat1, and possibly Stat2, with p48 in the IFN-α-induced ISGF3. As noted above, this type of protein-protein contact between dissimilar proteins bound to enhancer sites (23, 43) is probably important to the activities of the STAT family of proteins as it is to other families of transcription factors.

MATERIALS AND METHODS

Cell culture. Human U3A cells, U6A cells, and HeLa S3 cells were maintained in DMEM supplemented with 10% fortified bovine calf serum (Cosmic serum; HyClone). Transfection of cells with Stat cDNAs in expression vector pRcCMV (Invitrogen) was carried out by standard procedures (2, 16). Cells containing the plasmid were selected for resistance to G418 (500 μg/ml) and screened for

expression of Stat protein by immunoblot with Stat1 COOH-terminal antiserum (see below) and electrophoretic mobility shift assay with a Ly6E probe as described elsewhere (39). Both G418-resistant pools and independent clones were analyzed with similar results. IFN-γ (a gift from Amgen) was used at a concentration of 5 ng/ml; IFN-α (a gift from Hoffmann La Roche) was used at a concentration of 500 IU/ml. For phenotypic HAT selections, 5×10^4 cells from each cell line were plated and subjected to selection for 20 days in HAT medium (hypoxanthine, aminopterin, thymidine) (GIBCO BRL) containing IFN-α in the continued presence of G418, then stained with methylene blue.

Plasmid construction. Expression plasmid pRcCMV (Invitrogen) carrying Stat1 or Stat2 cDNA and their derivatives was used for all cell lines. The yeast bait plasmids were made in the yeast expression vector pEG202, and the prey plasmids were made in the yeast expression vector pJG4.5 (11). Inserts were generated by PCR amplification using Vent Polymerase (NEB) and verified by DNA sequencing. For glutathione *S*-transferase (GST) fusion protein expression, inserts were subcloned into the vector pGEX-5X-1 (Pharmacia).

Yeast interaction analysis. Yeast culture, transformation, and handling were as described elsewhere (2). For interaction assays, yeast transformants in strain EGY48 containing the bait plasmid, the galactose-inducible p48 COOH terminus prey plasmid, and the *lacZ* reporter plasmid, pSH18-34 (11), were selected on complete medium lacking uracil, histidine, and tryptophan (CM/UHT). The nucleotide sequences of all yeast expression vectors were verified prior to transformation. At least four independent colonies were patched onto a CM/UHT master plate, grown 24 h, then replica plated to four test plates with glucose (Glu) or galactose (Gal) carbon source. Activation of the chromosomal Leu2 reporter was analyzed on plates lacking uracil, histidine, tryptophan, and leucine. The *lacZ* reporter was analyzed by plating onto CM/UHT containing the chromogenic substrate X-Gal. Quantitative β-galactosidase assays in liquid culture were carried out as described elsewhere (2).

RNA analysis. Total RNA was prepared from confluent 3.5-cm dishes by using Trizol reagent (GIBCO BRL), digested with DNase I, and subjected to reverse transcriptase PCR (RT-PCR) analysis essentially as described elsewhere (46). RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamer primers. A mock reaction was carried out with no reverse transcriptase added (−RT). One-tenth of the resulting cDNA was used as template for 25 cycles of PCR in the presence of [α-^{32}P]dATP using specific primers for ISG15 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As a control for genomic DNA contamination, PCR was carried out with GAPDH primers using the mock (−RT) reaction products as templates. Following polyacrylamide gel electrophoresis, products were detected by autoradiography. Primer sequences are as follows: ISG15 a, 5′-CAACGAATTCCA GGTGTC-3′; ISG15 b, 5′-CCCTTGTTATTCCTCACC-3′; GAPDH a, 5′-GTG AAGGTCGGAGTCAAC-3′; GAPDH b, 5′-TGGAATTTGCCATGGGTG-3′.

Immunoblotting assays. For demonstration of tyrosine phosphorylation, U3A-derived cell lines were grown to confluence on 150-mm plates and treated with IFN for 15 min. Cell extracts were prepared as described elsewhere (39) and immunoprecipitated with a 1:100 dilution of rabbit antiserum to the Stat1 COOH terminus. The immune complexes collected on protein A-agarose (Oncogene Sciences) were washed four times with cell extraction buffer, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% gels and transferred to nitrocellulose filters. Phosphotyrosine was detected with monoclonal antibody 4G10 according to the manufacturer's directions (Upstate Biotechnology).

GST binding assays. Preparation of GST fusion proteins was carried out by induction of *Escherichia coli* containing the fusion vector at 30°C with 0.1 mM IPTG. Following lysis by sonication, GST proteins were purified on glutathione-Sepharose beads (Pharmacia) and washed extensively with phosphate-buffered saline (PBS). The proteins were left on the beads for affinity chromatography, or, for gel shift inhibition experiments, GST or GST fusion proteins were eluted from the beads with reduced glutathione (Sigma) and dialyzed against PBS. For in vitro translation of proteins, full-length Stat1, Stat2, or p48 cDNA was used to program coupled transcription and translation reactions in the presence of ^{35}S-labeled methionine according to the manufacturer's directions (TNT; Promega). GST binding assays were carried out as described elsewhere (2). The translation products were mixed with approximately 5 μg of GST or of each of the GST fusion proteins bound to glutathione-Sepharose beads in the presence of 1% nonfat dry milk as blocking agent. After washing, the specifically bound products were eluted by boiling in SDS-gel loading buffer and subjected to electrophoresis and autoradiography. For binding of HeLa cell extracts, cells were treated with IFN-γ for 16 h to increase the p48 content (21), and whole-cell extracts were exposed to GST or GST fusion protein beads. Eluted samples were subjected to SDS PAGE, and proteins were transferred to nitrocellulose filters, probed with p48-specific antiserum (Santa Cruz Biotechnology), and processed for chemiluminescence detection (Renaissance; Dupont).

DNA binding analysis. Nuclear extracts were prepared from HeLa cells that had been treated with IFN-γ for 16 h and IFN-α for 30 min by a standard protocol (18). GST and GST fusion protein concentrations were adjusted to ~250 ng/μl in PBS. Extracts were mixed with GST or various GST fusion proteins in gel shift buffer [20 mM HEPES (pH 7.9), 4% Ficoll, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 μg of poly(dI-dC)] for 15 min prior to addition of 0.5 ng of ^{32}P-labeled ISRE oligodeoxynucleotide (ISG15 promoter).

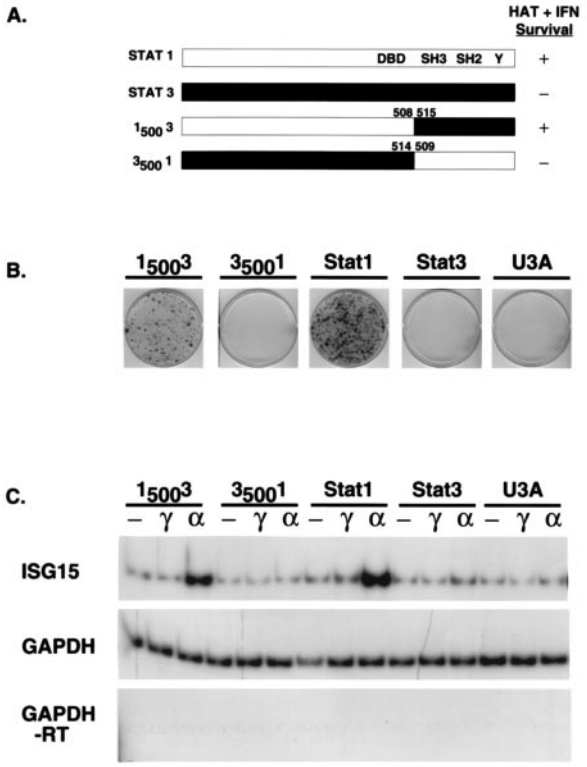

FIG. 1. IFN-α response requires the first 500 amino acids of Stat1 in Stat1:Stat3 chimeras. (A) Diagrammatic representation of the chimeras expressed in U3A cell lines and their ability to complement ISGF3-dependent survival in selective medium is indicated as + (growth) or − (no growth). (B) IFN-α-dependent survival of U3A cells and derived cell lines expressing the various Stat1 proteins indicated. (C) Analysis of endogenous mRNA induction by IFN treatment. U3A and U3A-derived cell lines were left untreated (−) or treated for 3 h with either IFN-γ (γ) (5 ng/ml) or IFN-α (α) (500 IU/ml). Reactions with primers specific for GAPDH or for the IFN-α-responsive gene ISG15 are indicated.

Fifteen minutes later, the mixture was separated on 5% polyacrylamide gels containing 0.25× Tris-borate-EDTA and detected by autoradiography.

RESULTS

Response to IFN-α requires the first 500 amino acids of Stat1. A genetically selected cell strain, U3A (29) lacks any Stat1 mRNA or protein and contains a stably integrated *E. coli* guanosine phosphoribosyl transferase (GPT) gene (to overcome aminopterin, a methyl transfer inhibitor) under control of an IFN-α-responsive promoter (27). U3A cells cannot survive in a selective medium (HAT medium containing IFN-α; see Materials and Methods) without being complemented by prior stable introduction of a Stat1 expression vector. When IFN-α is present, the induced Stat1 protein allows formation of ISGF3, leading to transcription and translation of *gpt*, allowing survival in HAT medium. In contrast, expression of Stat3, a closely related protein, results in no rescue of the IFN-α-dependent growth in HAT medium (Fig. 1A and B). This result accords with the fact that Stat3 does not produce ISGF3 even though Stat3 can be activated by IFN-α treatment and can dimerize, albeit weakly, with Stat2 (15). In studying U3A cells expressing hybrid Stat1:Stat3 molecules (16) we found that a hybrid containing the first ~500 amino acids of Stat1 and the remaining ~200 amino acids of Stat3 ($Stat1_{500}3$) was able to rescue cells in the HAT selection medium (Fig. 1A and B). The reverse construct, i.e., the first ~500 amino acids of Stat3

attached to the remainder of Stat1 ($Stat3_{500}1$), did not suffice to rescue the cells. These results suggested a specific function for Stat1 in IFN-α-dependent gene activation mapped to residues 1 to 500 in addition to the known dimerization regions between residues ~500 and 701 (the −SH2 region and phosphotyrosine residue [39, 40]). To test more directly for gene activation by the chimeric proteins, the induction of the IFN-α-responsive gene ISG15 was examined in the U3A cells expressing the various STAT constructs. ISG15 is immediately activated following interferon treatment through ISGF3 binding to an ISRE sequence (32). Neither Stat3 nor the $Stat3_{500}1$ hybrid allowed induction of ISG15 mRNA, while $Stat1_{500}3$ and Stat1 did induce the ISG15 gene (Fig. 1C).

A discrete region of Stat1 interacts with p48. One explanation for the requirement of the amino-terminal residues of Stat1 might be to provide contacts between Stat1 and the other ISGF3 components, p48 and Stat2. UV cross-linking experiments had shown that both Stat1 and p48 contacted the ISRE at neighboring sites (AGTTTN_3TTTCC [first two underlined T's contact p48; the next T contacts Stat1] [31]). The close proximity of the contacted bases on DNA suggested physical contact between these two proteins, and we therefore sought evidence of a Stat1-p48 interaction.

In the first experiments, a yeast two-hybrid system for detecting interaction was utilized (11). Several bait plasmids were constructed by fusing portions of Stat1 with the DNA binding domain of the bacterial protein LexA in the yeast expression vector pEG202. Since the amino-terminal half of p48 contains a DNA binding domain and the carboxyl-terminal half is required for formation of ISGF3 (44), we reasoned that the COOH half of the molecule might contain sites of interaction with other proteins. Therefore, a prey plasmid containing amino acids 200 to 393 of p48 fused to the "acid blob" transcriptional activation domain of the yeast expression plasmid pJG4.5 was constructed. Table 1 shows the results of the screening for interaction between various segments of Stat1 and the p48 COOH terminus. Transformation of the yeast host strain EGY48 along with a LexA-responsive *lacZ* reporter revealed that only the combination of the Stat1 amino acids 100 to 300 and the p48 COOH terminus satisfied the interaction criteria by activating both the Leu2 and LacZ reporters. Further subdivision of the region of Stat1 required for interaction with the p48 COOH terminus defined a region between amino acids 153 and 239. Truncation of this domain to amino acid 218 resulted in weak interaction compared to that observed with 153 to 239. To determine whether analogous segments of other STAT proteins showed evidence of interaction with p48, the regions of Stats 3, 4, 5, and 6 (residues ~150 to 250) were tested for their ability to mediate interaction in the yeast system. None of these protein segments showed interaction with p48, pointing to a high degree of specificity in the test system. We have not formally ruled out the possibility that differences in protein expression level in the yeast system account for the lack of detectable interaction, but multiple independent colonies were analyzed with the same results. However, a region of Stat2, the partner of Stat1 in the ISGF3, did interact with the p48 COOH terminus; in fact, a relatively short, 33-amino-acid stretch of Stat2 gave a positive interaction in the yeast system.

Mutations in the Stat1-p48 interaction domain impair IFN-α transcriptional responses. The amino acid sequences in the ~150-to-250 region of the various STAT family members show little exact amino acid conservation, but some general features are conserved. There is a predicted helical secondary structure, and a few conserved basic amino acids can be aligned at positions 150, 152, 161, and 175 in some or all of the family members (numbers are for Stat1). To test the importance of the conserved amino acids of this region of Stat1 in mediating the potential functional interaction between Stat1 and p48 within ISGF3, Stat1 cDNAs containing point mutations were prepared; the lysine residues at positions 150, 152, and 161 were individually changed to alanine. The altered Stat1 cDNAs were transfected into U3A cells, and stable cell lines were selected. The cells were subjected to the IFN-α-dependent HAT selection assay (Fig. 2A). The cell line containing a mutation at position 150, 3 amino acids away from the two-hybrid bait NH2-terminal boundary, had no effect on the complementation of U3A cells, yielding abundant colonies indistinguishable from cell lines with wild-type Stat1. The cell line with a mutation at position 152 (the N-terminal boundary of the segment used in the two-hybrid system) caused a distinct reduction in both the number and the size of colonies. Most striking was the result that cells bearing Stat1 with a mutation at residue 161 failed to survive the HAT selection assay. Microscopic examination of plates with these mutant cells showed only microcolonies (8 to 15 cells) after 20 days, a time when the wild-type and mutant $K_{150}A$ plates were nearly confluent. To test the correlation of the HAT selection phenotype with IFN-α-dependent transcription, ISG15 mRNA levels were examined (Fig. 2A). Cells containing the mutant $K_{150}A$ responded to IFN-α by accumulating ISG15 mRNA as did wild-type cells, while both mutants $K_{152}A$ and $K_{161}A$ failed to accumulate mRNA in response to IFN-α stimulation to the same extent as wild-type or mutant $K_{150}A$. No effect was observed on transcription of the IFN-γ-responsive gene, *IRF1*, with the mutants (data not shown). These results indicate an essential role of

TABLE 1. Activation of yeast two-hybrid reporters by STAT interactions with p48

Amino acids	Leu2[a]		LacZ[b]	
	Glu	Gal	Glu	Gal
Stat1				
1–109	−	−	−	−
107–279	−	+	−	+
185–279	−	−	−	−
289–374	−	−	−	−
383–487	−	−	−	−
107–156	−	−	−	−
107–189	−	−	−	−
107–244	−	+	−	+
153–189	−	−	−	−
188–244	−	−	−	−
188–279	−	−	−	−
153–244	−	+	−	+
153–239	−	+	−	+
153–218	−	+/−	−	+/−
Stat2				
156–189	−	+	−	+
155–261	−	+	−	+
Stat3, 155–249	−	−	−	−
Stat4, 154–245	−	−	−	−
Stat5, 155–261	−	−	−	−
Stat6, 128–244	−	−	−	−

[a] +, growth of complete patch on leucine-deficient medium 48 h after replica plating; −, no growth on leucine-deficient medium after 48 h; +/−, slow growth on leucine-deficient medium (incomplete patch after 48 h).

[b] +, readily detectable blue color on X-Gal-containing medium 48 h after replica plating; −, no detectable blue color on X-Gal-containing medium after 48 h; +/−, faint blue color on X-Gal-containing medium 48 h after replica plating.

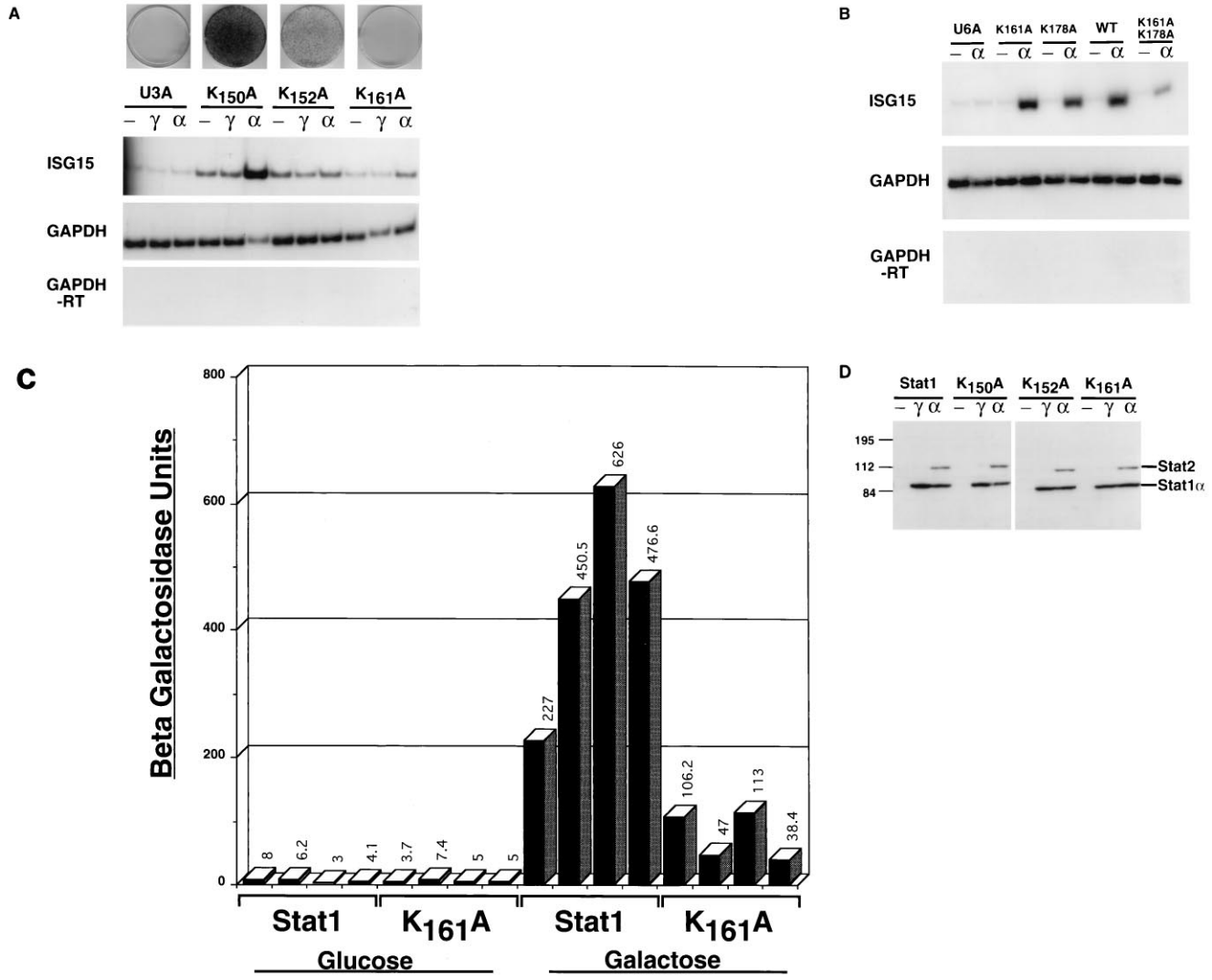

FIG. 2. Mutations in Stat1 inhibit IFN-α response by decreasing affinity for p48. (A) (Top) IFN-α-dependent survival of U3A cells and derived cell lines expressing the wild-type or mutated Stat1 proteins. (Bottom) Analysis of endogenous mRNA induction by IFN treatment in U3A-derived cell lines. Manipulations were as described for Fig. 1. (B) Effect of mutations in Stat2 on IFN-α-dependent transcriptional induction. Analysis of endogenous mRNA induction in U6A and derived cell lines expressing various Stat2 proteins after 3 h of treatment with IFN-α. (C) Stat1 mutation $K_{161}A$ results in decreased affinity for p48 in the yeast system. Wild-type (Stat1) or mutant ($K_{161}A$) bait plasmids (amino acids 153 to 244) were used to transform host strain EGY48 along with the galactose-inducible p48 COOH terminus prey plasmid and the pSH18-34 *lacZ* reporter. Extracts of four clones expressing either Stat1 or the $K_{161}A$ mutant were assayed for β-galactosidase activity (2) after 4 h in growth medium containing glucose or galactose as indicated. (D) Mutated Stat1 proteins are activated and dimerize in response to IFN-γ and IFN-α. An anti phosphotyrosine immunoblot of U3A-derived cell lines following immunoprecipitation with Stat1-specific antiserum is shown. U3A cells expressing wild-type Stat1, $K_{150}A$, $K_{152}A$, and $K_{161}A$ were left untreated (−) or treated for 15 min with 5 ng of IFN-γ (γ) or 500 IU of IFN-α (α) per ml. Positions of molecular weight standards, Stat1α, and Stat2 are indicated.

lysine residue 161 of Stat1 in the IFN-α transcriptional response.

To determine that the mutant STAT proteins could still respond to interferon activation, the individual cell lines expressing the proteins were treated with IFN-γ or IFN-α. Cell extracts were precipitated with anti-Stat1 antiserum and tested in two ways. First, the IFN-γ treatment was shown to cause phosphorylation on tyrosine approximately equally to the wild type for each lysine mutant. In addition, Stat1 precipitates from IFN-α-treated cells were also tested for coprecipitation of tyrosine-phosphorylated Stat2. Again, the mutant proteins performed identically to wild-type protein (Fig. 2D).

We next tested interactions between Stat2 and p48. A similar but smaller region of Stat2 was found to mediate p48 interactions in the yeast two-hybrid system, and the functional importance of this interaction was similarly tested by mutagenesis. Mutations were engineered into Stat2 cDNAs changing the conserved lysine residue at position 161 (this conserved lysine appears at the same numerical position in Stat1 and Stat2) to alanine. Because a conserved lysine residue was also found at position 178 of Stat2, it was also substituted with alanine. Stat2 cDNAs containing both individual and double amino acid substitutions were used to transfect the U6A cell line, which is defective in Stat2 expression (20), and cell lines stably expressing the mutated Stat2 proteins were selected. Cell lines containing the single-site lysine substitutions (at residue 161 or 178) supported ISG15 mRNA accumulation in response to IFN-α treatment to a similar extent as cell lines reconstituted

with wild-type Stat2 (Fig. 2B). When the Stat2 contained both mutations, it was also capable of supporting IFN-α-inducible transcription of ISG15, but the mRNA accumulation was markedly reduced compared to the other U6A cell lines.

Mutation of Stat1 residue K161 directly inhibits p48 interaction. To test whether the Stat1 protein with the mutation $K_{161}A$, which inhibited the IFN-α transcriptional response, was defective in the interaction with p48, the DNA encoding the mutated region from amino acid 153 to 244 was subcloned into the two-hybrid bait plasmid and yeast cells were transformed and subjected to the interaction tests. The mutated Stat1 domain was capable of interacting only weakly with the p48 prey compared to the wild-type domain. Quantitative β-galactosidase assays demonstrated that the interaction was approximately fivefold weaker than that of the wild-type Stat1 domain (Fig. 2C). Thus, all evidence indicates that the residues from 153 to 244 form a functional interaction domain between Stat1 and p48 which is required for IFN-α stimulation of transcription. The Stat2 domain containing the double mutation $K_{161/178}A$ was also tested in quantitative β-galactosidase assays, but no clear difference was observed in interaction-dependent β-galactosidase accumulation (data not shown), possibly due to the retained interaction capability of this mutant.

Characterization of STAT and p48 contacts in solution. To further support the two-hybrid interaction trap data and somatic-cell genetic results, in vitro biochemical evidence of a Stat1-p48 interaction was sought. GST fusion proteins with the $Stat1_{153–244}$ and $p48_{200–393}$ regions were expressed in *E. coli* and purified on glutathione-agarose beads. Because of the apparent interaction in the yeast system of the $Stat2_{156–188}$ fragment with p48, it was also prepared as a GST fusion product. These affinity reagents were incubated with in vitro-translated, ^{35}S-labeled full-length Stat1, Stat2, or p48 protein (Fig. 3A). Separation of the bound polypeptides by SDS-PAGE followed by autoradiography revealed that GST-STAT domain fusions were capable of binding to p48 in solution while GST alone did not. In fact, the Stat2 domain appeared to react more strongly with p48 than the Stat1 domain. In the complementary experiment, GST-p48 was capable of binding to both full-length in vitro-translated Stat1 and full-length in vitro-translated Stat2. Another GST-STAT affinity experiment was carried out in which HeLa cells were the source of the p48 protein (Fig. 3B). Nuclear extracts from HeLa cells were incubated with the fusion protein beads carrying the Stat1 or Stat2 fragment and then subjected to SDS-PAGE and immunoblotting with p48-specific antiserum. Both GST-Stat1 and GST-Stat2 fusion proteins were capable of binding to p48 from the HeLa cell extracts, while GST alone did not. Thus, both the in vitro interaction and the yeast interaction assay indicated physical contact between the COOH terminus of p48 and Stat1 and -2.

STAT-p48 interaction domains inhibit ISGF3 formation. As a final test of interactions between Stat and p48 proteins, we carried out in vitro experiments to try to disrupt ISGF3 formation by fragments of the participating proteins, a commonly used test for protein-protein interaction between DNA-binding proteins (10, 39). Fragments of Stat1, Stat2, and p48 as GST fusion proteins were prepared by elution from the beads and mixed with HeLa cell extracts from IFN-α-treated cells in the absence of radiolabelled ISRE DNA (Fig. 3C). Fragments of each of the constituents of ISGF3 were capable of preventing ISGF3 formation upon addition of radiolabelled ISRE DNA. The p48 fragment was the most effective at blocking ISGF3 formation followed by Stat1 and then Stat2. In contrast with the affinity precipitation experiments, in this assay the Stat2 fragment was found to be less active at binding p48. It is possible that the protein is denatured during elution from the glutathione beads or that the large GST carrier portion prevents accessibility to the Stat2 sequences. An alternate explanation is that the Stat2 which is already present in the extract is tightly bound to p48 and the exogenously added Stat2 fragment did attach to its binding site. Perhaps most important to assure specificity of the competition, the mutant $K_{161}A$ protein fragment did not prevent ISGF3 formation to the same extent as the wild-type counterpart. Experiments in which the fusion proteins were added after ISGF3 complex was allowed to assemble on the DNA showed the STAT fragments to be ineffective at dispersing the already formed complexes (Fig. 3D). However, the COOH-terminal p48 fragment was capable of disrupting already-formed ISGF3, although not as effectively as when it is added before the DNA probe. Apparently, as the DNA-stabilized factor is formed, the interaction sites become inaccessible to exogenously added protein competitors.

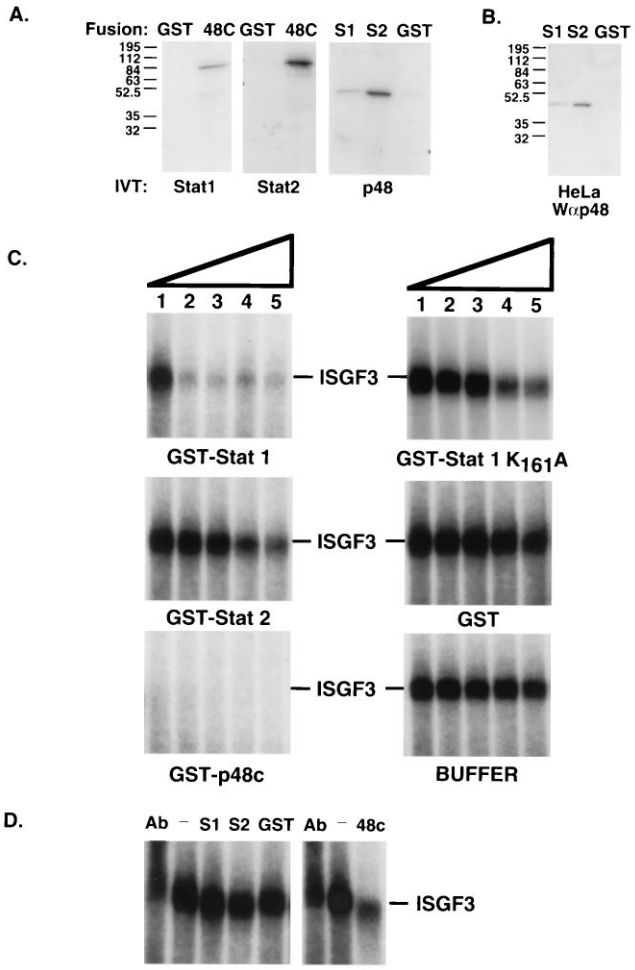

FIG. 3. Interaction of Stat and p48 domains in vitro (A) Autoradiogram of ^{35}S-labeled in vitro translation products (IVT) selected by GST affinity columns with fused Stat1 (S1) (amino acids 153 to 244), Stat2 (S2) (amino acids 156 to 189), or p48 (48c) (amino acids 200 to 393). (B) Immunoblot of affinity-precipitated p48 from HeLa cell extracts. Molecular size markers are in kilodaltons. (C) ISGF3 electrophoretic gel mobility shift interference by STAT domains. Amounts of ~250-ng/μl solutions of each protein added to the reaction mixtures are indicated at the top. Control reaction mixtures (buffer) contained mock eluted samples with no protein. (D) Same as panel C except that ISGF3 was allowed to form on the ISRE oligodeoxynucleotide prior to addition of 5 μl of eluted GST or GST fusions with the Stat1 (S1), Stat2 (S2), or p48 (48c) interaction domains defined. Ab, incubation with p48-specific antiserum; −, no added protein.

DISCUSSION

There is a growing appreciation that transcriptional activators act in concert with other proteins to effect transcriptional increases (25). For the STAT proteins that seemed likely, for their original isolation was as part of a complex in which both Stats 1α and 1β, Stat2, and p48 were all present. To more fully understand the IFN-α-induced ISGF3 complex and to begin to assign function to previously unassigned regions of the Stat molecule were the aims of the experiments reported here.

We conclude that in the ISGF3 complex physical contacts between Stat1 and p48 definitely exist and are required for ISGF3 function. The amino acids important for this contact lie between residues ~153 and 239 of Stat1 and the COOH half of p48 (residues 200 to 393). No function had been assigned previously to this region of a Stat protein, and we now refer to this region as ID1 (interaction domain 1). Initial success in detecting this interaction depended on the yeast two-hybrid system demonstrating interactions between protein fragments that did not require tyrosine phosphorylation. Previous attempts at mutagenic analysis of Stats 1 and 2 in mammalian cells were made difficult by the requirement for tyrosine phosphorylation to study subsequent interaction (17, 30). The contacts between Stat1 and p48 may be transient in the DNA-free state in vivo but are essential to IFN-α-induced transcriptional activity after Stat1:2 translocation to the nucleus. These contacts likely are necessary for the stabilization of the entire DNA binding complex because mutations within ID1 which weaken the interaction with p48 also abrogate IFN-α-dependent transcriptional activity.

Our results are consistent with experiments in which overexpression of Stat1 and p48 allowed detection of DNA binding by a Stat1 homodimer plus p48 (4). Similar overexpression experiments with U3A cells lacking Stat1 suggest both that Stat2 has a weak capacity to dimerize and that contact between Stat2 dimers and p48 can occur (3).

Both the yeast system and the in vitro assays of interaction presented here suggest that a region of Stat2 is also capable of contacting p48. It is possible that Stat2 also interacts functionally with p48 in ISGF3-dependent transcription through an ID1 functional domain. Mutations in Stat2 targeting lysine residues analogous to those which decrease the affinity of Stat1 for p48 did not have the same effect in reducing the IFN-α transcriptional response. A double mutant of Stat2 containing two substitutions reintroduced into U6 cells lacking wild-type Stat2 did result in a decreased IFN-α response, suggesting a less central role for the Stat2-p48 association in the ISGF3 complex. In the in vitro solution binding experiments, Stat2 consistently bound better to p48 than Stat1. It is possible that the highest affinity interaction is between Stat2 and p48 but that Stat1 has a higher affinity for the DNA sequence that neighbors the p48 binding site so that Stat1-p48 contacts are more prominent in the ISGF3 transcription factor.

We also found that preformation of ISGF3 on DNA substantially stabilized the complex to competition with GST fusion proteins. The stability was likely achieved by the multivalent interactions which take place on the ISRE. It is possible that multiple STAT-p48 contacts exist so that each protein potentially touches the other two. As Stat1 and p48 both make base-specific DNA contacts, the complex can hold together even better on the ISRE DNA. The exact oligomeric structure of p48 and the stoichiometry of the proteins in ISGF3 are still not known, but it is clear that Stat1 and Stat2 interact most likely as a heterodimer (31) and that Stat2, and not Stat1, furnishes the obligatory transactivation domain of ISGF3 (27, 30). There are three possible models which can allow for Stat1 alone or both Stat1 and Stat2 to contact p48 in the complex (Fig. 4). The first possibility is that p48 is a dimer in the complex. In this model, both Stats could have easy access to binding sites on p48. Although it has been shown that the IRF family members ICSBP and IRF-1 and -2 can form complexes in the presence and absence of DNA (5, 35), we do not necessarily favor this model for ISGF3 because the ISRE (AGT TTNNNTTTCC) does not have the symmetry requisite for dimer binding and the UV cross-linking data shows specific binding of the ISRE by p48 on only one half of the element (31). In a second model, only one p48 is bound to the ISRE, but both Stat1 and Stat2 touch the p48 molecule. Two STAT molecules cannot contact precisely the same site on the p48; this model would call for two separate binding sites on the 193 amino acids of the p48 COOH terminus used in the yeast experiments. The mutational analysis of Stats 1 and 2 can be interpreted as supporting this model since lysine 161 of Stat1 is critical for p48 contact while the analogous site of Stat2 can be mutated without consequence. While the interaction domains of the two Stat proteins are similar, they do not share a single partner site on p48. The final model is more reductionist: the ISRE binds a monomer of p48, and this monomer only is bound by the Stat1 protein. This view of ISGF3 assembly would accommodate both Stat1 and Stat2 as a heterodimer with Stat2 and its transactivation domain being carried along into ISGF3 by association with Stat1 only. It will be necessary to examine the p48 and ISGF3 oligomeric structure in more detail to map the amino acids in p48 which are required for interaction with the Stats in order to establish the essentials of ISGF3 formation.

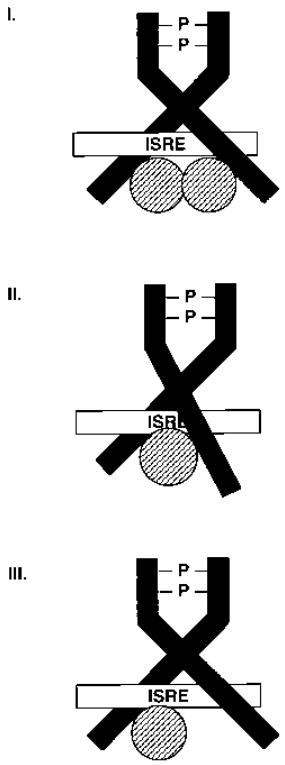

FIG. 4. Models of protein-protein interactions in the ISGF3 complex. Solid bar, Stat1; shaded bar, Stat2; circle, p48; open box, ISRE-containing DNA; P, SH2-phosphotyrosine dimerization interface. See the text for details.

The protein family that includes p48 has at least five other mammalian members (IRF-1, IRF-2, ICSBP, IRF3, and IRF4

[1, 7, 12, 24, 26]) which share homology in the DNA binding domain as well as some conservation of carboxyl-terminal sequences. It is tempting to think that some of these proteins might interact with some of the other STAT molecules (Stats 3 to 6). We found the p48 interaction to be specific for Stat1 and Stat2 and are currently testing other possible pairwise interactions between the two protein families. If other ISGF3-like factors exist which bind to distinct response element sequences, this could greatly increase the diversity of transcriptional activation from the STAT family. It is possible that these factors exist but we do not know which elements they bind to; further study of promoter regions of immediate-response genes is called for. Enhancer binding proteins appear to operate maximally only by discrete interaction with both general and specific transcription factors which also bind to the promoter DNA. In the enhanceosome complex of the IFN-β promoter, the IRF family has a critical role in mediating interactions with the activated transcriptional regulator NF-κB (28). It is reasonable to expect the other IRFs to have similar essential structural relationships to other promoter bound factors.

Recent reports have indicated that Stat1 and Stat3 may interact with other protein factors (e.g., c-Jun [34] and SP1 [22]), and compound arrays of transcription factor binding sites have been reported for STAT-responsive promoters (e.g., c-fos (14, 33). It will be interesting to determine if ID1 is utilized for those contacts as well as for the Stat1-p48 contact. In general, it seems highly likely that multiple interactions between STATs and the various proteins active in transcription can be anticipated.

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