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Function of Stat2 Protein in Transcriptional Activation by Alpha Interferon

SAJJAD A. QURESHI,[1] STEWART LEUNG,[2] IAN M. KERR,[3] GEORGE R. STARK,[2] AND JAMES E. DARNELL, JR.[1]*

Laboratory of Molecular Cell Biology, The Rockefeller University, New York, New York 10021[1]; Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195[2]; and Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom[3]

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Alpha interferon (IFN-α)-induced transcriptional activation requires the induction of a complex of DNA-binding proteins, including tyrosine-phosphorylated Stat1 and Stat2, and of p48, a protein which is not phosphorylated on tyrosine and which comes from a separate family of DNA-binding proteins. The isolation and characterization of U6A cells, which lack Stat2, have allowed the introduction of normal and mutant forms of Stat2 so that various functions of the Stat2 protein can be examined. As reported earlier, Stat1, which is the second target of tyrosine phosphorylation in IFN-α-treated cells, is not phosphorylated in the absence of Stat2. We show that all mutations that block Stat2 phosphorylation also block Stat1 phosphorylation. These include not only the mutations of Y-690 and SH2 domain residues that are involved in tyrosine phosphorylation but also short deletions at the amino terminus of the protein. Two mutants of Stat2 that are not phosphorylated on tyrosine can act as dominant negative proteins in suppressing wild-type Stat2 phosphorylation, most likely by competition at the receptor-kinase interaction site(s). We also show that the COOH-terminal 50 amino acids are required for transcriptional activation in response to IFN-α. Mutants lacking these amino acids can be phosphorylated, form IFN-stimulated gene factor 3, and translocate to the nucleus but cannot stimulate IFN-α-dependent transcription. Seven acidic residues are present in the deleted COOH-terminal residues, but 24 acidic residues still remain in the 100 carboxy-terminal amino acids after deletion. Thus, transcriptional activation is unlikely to depend on acidic amino acids alone.

Transcriptional activation in response to alpha interferon (IFN-α) is mediated through the activation of a multiprotein DNA binding complex described as IFN-stimulated gene factor 3 (ISGF3) (14). This factor was purified from the nuclei of IFN-α-treated cells, and cDNAs encoding its four constitutive proteins, nominally with sizes of 113, 91, 94, and 48 kDa, were cloned (4, 5, 25, 29). The three larger proteins were the first recognized members of the growing family of signal transducers and activators of transcription (STAT) proteins (2, 24). The cDNAs encoding the 91- and 84-kDa proteins, which were sequenced first, arise from differential splicing of primary transcripts from the same gene and are referred to as Stat1α and -β. The sequence of the cDNA for the 113-kDa protein, now referred to as Stat2, revealed blocks of amino acid identity with the sequence of Stat1, and many of these blocks have proved to be conserved in all of the six presently known STATs. The region of highest amino acid conservation is in the SH2-like region between residues 550 and 700 in each of the proteins. The SH2 groups are necessary, first, to dock the STAT on a phosphorylated residue on the receptor chain (6, 7). After STAT phosphorylation on tyrosine that is catalyzed by a receptor-associated JAK (Janus) kinase (30, 32), the Stat1α or -β proteins form a heterodimer with Stat2 (20, 26). Stat1 homodimers can also form after IFN-α treatment, though not so abundantly as after IFN-γ treatment, during which Stat2 is not activated (27). The final member of the ISGF3 complex, the 48-kDa protein, is a member of a different group of DNA-binding proteins with homology to IFN response factor 1 (IRF-1) (29), the first member of the group to be cloned (16). It has been shown recently by UV cross-linking analysis of ISGF3 with the IFN-α-stimulated response element (ISRE) that both the 48-kDa protein and either Stat1α or -β contact DNA at specific residues in the ISRE when ISGF3 is bound to the element (20).

Cell lines that lack Stat1 but into which wild-type and mutant Stat1 protein can be introduced have been extremely useful in allowing the exploration of the molecular anatomy of that protein (17). For example, the necessary role for a single target tyrosine at residue 701 which, after phosphorylation, participates in dimerization via intermolecular -SH2 phosphotyrosine interaction prior to DNA binding was defined (27). In addition, U3A cells have allowed (i) the demonstration that residues 400 to 500 specify DNA site recognition and probably provide contact sites with DNA (9), (ii) demonstration of the importance of the SH2 group in determining which receptor will activate the STAT molecule (7), and (iii) demonstration of a requirement for the terminal 38 amino acids of Stat1α that are missing in Stat1β for adequate IFN-γ transcriptional response (17). It was noted with U3A cells that either Stat1α or -β would form ISGF3 and activate genes in response to IFN-α, which is in contrast to the results with IFN-γ (17). Recent isolation and characterization of a new mutant cell line, U6A, that lacks Stat2 (the 113-kDa component of ISGF3 [13]) has now allowed the examination of the functional regions of the Stat2 molecule. In the present experiments, we have examined the effects of Stat2 mutations on phosphorylation of tyrosine on both Stat1 and Stat2, the heterodimerization of Stat1 and -2, translocation to the nucleus, binding to DNA in concert with the p48 protein, and IFN-α-dependent gene activation.

MATERIALS AND METHODS

Cell lines and preparation of cell extracts. 2fTGH (18), 2fTGH clone U6A (13) and its derivatives, and WI-38 VA13 subline 2RA human fibroblasts (ATCC

* Corresponding author. Phone: (212) 327-8791. Fax: (212) 327-8801. Electronic mail address: darnell@rockvax.rockefeller.edu.

FIG. 1. Amino acid sequence of Stat2. The full-length Stat2 cDNA clone used in the studies described in this paper encodes a protein of 851 amino acids (5). Amino acids 130 to 290 of Stat2 have the potential to form three alpha helices (helices 1 to 3). A set of repeated leucines within helix 2 (open boxes) could form a leucine zipper. The carboxy terminus (amino acids 704 to 830) of Stat2 has a high concentration of acidic amino acids (29 of 127 residues), which is often found in protein regions involved in the transactivation of genes. Amino acids 499 to 556 share homology with the Src homology 3 domain (SH3 domain), and amino acids 572 to 667 share homology with the Src homology 2 domain (SH2 domain). R-601 is a conserved residue present in the SH2 phosphotyrosine binding site, and Y-690 is the single Y phosphorylated upon ligand activation of Stat2. Numbers above the Stat2 sequence show the positions of substitutions (boldface) described in this paper. Arrows indicate the positions of amino-terminal deletions, and asterisks indicate the positions of stop codons introduced to generate the carboxy-terminal deletions.

CCL 75.1) were maintained in Dulbecco's modified Eagle's medium plus 10% supplemented calf serum (Hyclone Laboratories). Nuclear and whole-cell extracts were prepared as described previously (14, 20).

Stat2 expression vector and mutagenesis of Stat2 protein. To express wild-type and mutant Stat2 in U6A cells, a Stat2 cDNA (5) was cloned into eukaryotic expression vector pMNC (19). Mutations in the Stat2 protein were created by standard PCR techniques and oligonucleotide-primed mutagenesis. All mutations were confirmed by sequencing. To establish permanent cell lines, the expression vector (pMNC) containing either full-length Stat2 or mutant versions of Stat2 were introduced into U6A cells by calcium phosphate transfection procedures as described previously (19). G418-positive colonies were ring cloned and characterized by Western blotting (immunoblotting) for the expression of an appropriate protein(s). Positive cell lines were then expanded and used in subsequent experiments.

Immunoprecipitation and Western blotting. Protein immunoprecipitation and Western blotting of STAT proteins have been described previously (25). Stat1c, Stat2n, and Stat2c antibodies (5, 20, 25) were used for immunoprecipitation and Western blot detection of Stat1 or Stat2 protein. For detection of tyrosine-phosphorylated proteins, membranes were probed with anti-phosphotyrosine antibody 4G10 (UBI). When required, filters were stripped of previous antibody by incubating them in strip buffer (62.5 mM Tris-HCl [pH 6.7], 2% sodium dodecyl sulfate [SDS], and 100 mM β-mercaptoethanol) at 50°C for 30 min.

Transient transfections and assay for reporter gene activity. pISRE-LUC, which expresses a luciferase gene under the control of a single copy of an ISRE from the ISG15 gene (21), was used to score the gene activation induced by IFN-α. To assay the transcription induction by IFN-α, pISRE-LUC was transfected into a human fibroblast cell line (WI-38) by the calcium phosphate transfection procedure described previously (19). Briefly, cells were exposed to DNA-$CaPO_4$ precipitates for 16 to 20 h, after which the precipitates were removed by washing the cells twice with warm phosphate-buffered saline and split equally into two plates. The transfection mixture contained a total of 20 μg of DNA per ml (4 μg of pISRE-LUC, 4 μg of pMNC or pMNC containing various Stat2 cDNAs, 1 μg of RSVβGAL, and pBluescript as carrier DNA). At 40 h post-transfection, one plate was treated with IFN-α (500 U/ml) for 6 h while the other was left untreated. Cells were then lysed on the plate in 400 μl of lysis buffer (25 mM Tris [pH 7.8], 2 mM dithiothreitol, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid [CDTA], 1% Triton X-100, and 10% glycerol). Luciferase activity in the cell extracts was determined according to the manufacturer's instructions (Promega) and was normalized by assaying the β-galactosidase activity in the same extracts (23).

EMSA. An electrophoretic mobility shift assay (EMSA) using an ISRE from the ISG15 gene (22) or an IFN-γ activation site (GAS) from the LyE/6 gene (11) was performed as described previously (15, 28).

RNA preparation and RNase protection assay. For RNA preparation, the cell lines were treated with IFN-α or IFN-γ for 4 h prior to the harvesting of the cells. Total RNA was prepared, and RNase protection experiments were performed as described previously (13). The probes used were 6-16 (protects 190 bases) (1); ISG54 (250 bases from exon 2), kindly provided by D. Levy; IRF-1 (175 bases); and γ-actin (130 bases) (17).

Preparation of figures for publication. For the preparation of Fig. 2, 4, 5, and 6, we used a Flatbed Scanner (ScanJet IIcx-Hewlett Packard) to scan the images from the autoradiograms and desktop publishing software (Adobe Photoshop version 3.0 [Adobe Systems Inc., Mountain View, Calif.] and Canvas version 3.5 [Deneba Systems Inc., Miami, Fla.]) to produce figure composites in the final form as they appear in this publication.

RESULTS

A number of deletion and substitution mutations of Stat2 DNA were prepared in an expression vector so that they could be reintroduced into permanently transfected U6A cells to test the functional consequences of the mutant proteins. The sequence of Stat2 and the sites of the mutations that were reintroduced into U6A cells as permanent transfectants are given in Fig. 1. The original experiments with U6A cells had uncovered an unexpected requirement for an early protein-protein interaction in the IFN-α-dependent formation of ISGF3. Both Stat1 and Stat2 are phosphorylated on tyrosine in response to IFN-α, but in U6A cells lacking Stat2, no phosphorylation of Stat1 occurred (13). This result contrasted with the result with U3A cells which lacked Stat1 protein but in which Stat2 activation by tyrosine phosphorylation was normal (10). Therefore, the first mutations of Stat2 to be analyzed were those concerned with the cytoplasmic phosphorylation of this protein and the subsequent effects on Stat1 phosphorylation and heterodimer formation, the first steps in ISGF3 formation. Permanent U6A cell derivatives expressing wild-type or mutant Stat2 protein in amounts approximately equal to those expressed by the parental 2fTGH cell line were used in the

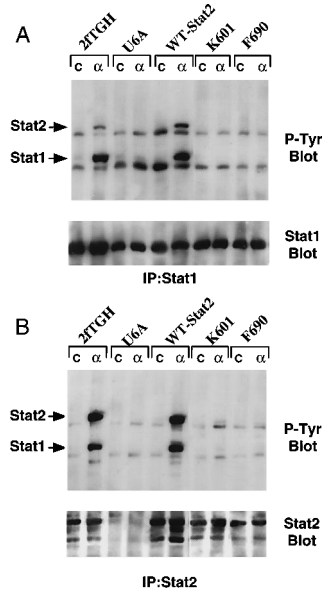

FIG. 2. Phosphorylation of Stat1 and Stat2 wild-type and mutant proteins in U6A cell derivatives. Whole-cell lysates of cells that were either left untreated (c lanes) or treated with IFN-α (α lanes) for 45 min were immunoprecipitated with Stat1 antiserum (A) or Stat2 antiserum (B). The immunoprecipitates (IP) were fractionated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose filters that were first probed with antiphosphotyrosine antibody (4G10; Upstate Biotechnology) (P-Tyr Blot) and subsequently with Stat1c (Stat1 Blot) or Stat2n (Stat2 Blot) antibody. The cell lines are labeled at the tops of the lanes. 2fTGH, the parental cell line used to generate U6A cells, expresses wild-type Stat2 protein. The U6A cell line lacks Stat2 protein. WT-Stat2 is U6A complemented with a full-length Stat2 cDNA. K601 expresses Stat2 R-601→K-601, and F690 expresses Stat2 Y-690→F-690.

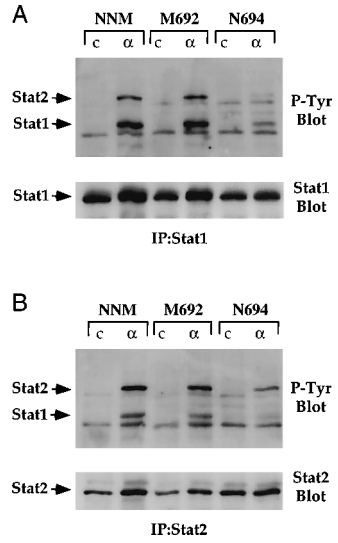

FIG. 4. Tyrosine phosphorylation of further Stat2 mutants. Stat1 and Stat2 wild-type and mutant proteins were analyzed as described in the legend to Fig. 2. The results given are from cell lines expressing Stat2 with point mutations at amino acids 687 to 689 (NMM), 692 (M692), and 694 (N694), with Stat1c (A) and Stat2n (B) antibodies being used.

experiments before (Fig. 2, lanes c) and after (Fig. 2, lanes α) IFN-α treatment. Cell extracts were precipitated with either anti-Stat1 or anti-Stat2 antibodies. The precipitates were separated by electrophoresis, blotted to nitrocellulose, and tested for tyrosine phosphorylation and subsequently for total protein (Stat1 and Stat2 are shown by arrows in Fig. 2; nonspecific bands were encountered after precipitation with either antiserum, but they did not obscure the visualization of specific bands).

Tyrosine 690 is known to be the single residue phosphorylated in Stat2 after IFN-α treatment (10), and residues 570 to 670 encode the SH2 domain, which plays a directive role in interaction with the receptor kinase complex where tyrosine phosphorylation occurs (7). The Stat2 mutant Y-690→F-690 was not tyrosine phosphorylated, and mutation of R-601→K-601, which in the SH2 crystallographic structure is the crucial residue in the crevice that binds phosphotyrosine (31), also prevented IFN-α-induced tyrosine phosphorylation of Stat2 (Fig. 2B). Both of these mutations in Stat2 also prevented the IFN-α-induced phosphorylation of Stat1 (Fig. 2A). IFN-γ-induced tyrosine phosphorylation of Stat1 was normal in U6A cells or U6A cells with these two Stat2 mutations (data not shown). While the basis of the requirement for Stat2 in the phosphorylation of Stat1 remains unknown, there are two possible protein interactions that might be involved in Stat2-dependent Stat1 activation. First, the assembly of a receptor kinase complex competent to attract and/or phosphorylate Stat1 might require a phosphorylated Stat2 molecule. Second, Y-690 on the Stat2 molecule might itself be the docking site for Stat1 to bind to the receptor kinase complex. To shed light on these two possibilities, we sought to assay mutant Stat2 proteins for their ability to interfere (act as "dominant" negative mutations [8]) with a wild-type Stat2.

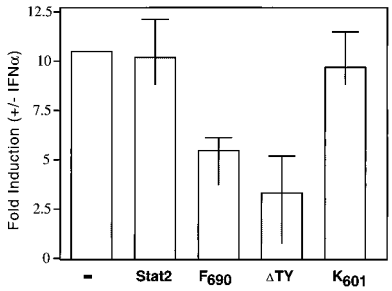

FIG. 3. Effect of Stat2 mutations on ISRE-dependent luciferase activity. Plasmids carrying a luciferase gene under the control of an ISRE and pMNC or pMNCplus Stat2 (wild type or mutant) were transiently transfected into WI-38 cells (a human fibroblast cell line). At 48 h after transfection, half the cells were treated with IFN-α for 6 h and half were not treated; cell lysates were prepared, luciferase activity was determined, and fold induction was determined by dividing treated luciferase activity by untreated luciferase activity. Fold increases in luciferase activity as the means of three experiments are plotted for vector alone (−), vector with WT-Stat2 (Stat2), and K601, F690, and ΔTY (a Stat2 mutant from which amino acids 614 to 712 have been deleted) mutants.

A luciferase reporter construct containing an ISRE, the binding site of IFN-α-activated ISGF3 (15), was constructed, and acute transfection of a fibroblast line was carried out. Following IFN-α treatment, luciferase expression was measured. There was a ligand-dependent 8- to 10-fold induction of luciferase in cell lines with no extra Stat2 transfected or with transfected wild-type Stat2 (Fig. 3 [– and Stat2 bars]). Simultaneous cotransfections of the reporter with vectors expressing various mutations of Stat2 were then carried out to test for dominant negative effects (Fig. 3). Expression of Stat2 Y-690→F-690 reduced the IFN-α response by about 40 to 60%. A deletion mutant, ΔTY, which lacks 98 amino acids (residues 614 to 712), including the Y-690 site, plus about 50 amino acids from the COOH end of the SH2 domain produced an even more drastic suppression (60 to 80%) of the IFN-α response. The expression of Stat2 R-601→K-601, which disables -SH2 in the binding of phosphotyrosine, had no effect on IFN-α induction in this assay system. These results are in accord with the following

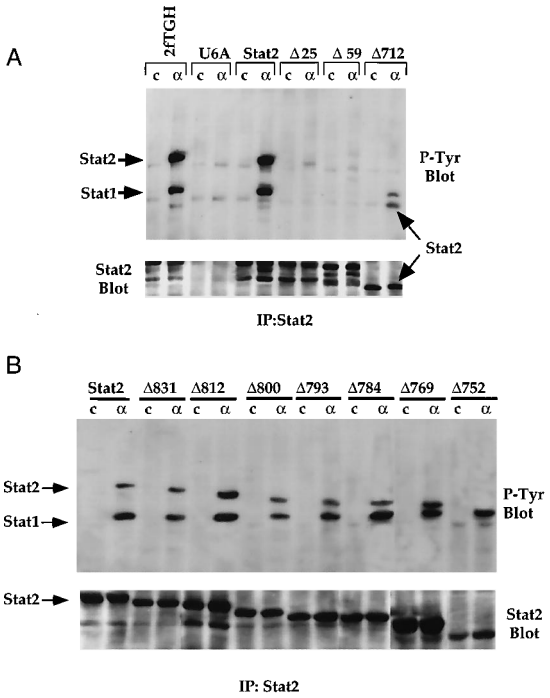

FIG. 5. Effect of deletion of Stat2 from the amino and carboxy termini. Immunoprecipitation and Western blot analysis of Stat1 and Stat2 proteins were performed as described in the legend to Fig. 2. 2fTGH, U6A, and Stat2 were as described in the legend to Fig. 2; Δ25 and Δ59 had deletions of the first 25 and 59 amino acids, respectively, from the amino terminus of Stat2, and Δ712 had a stop codon inserted after amino acid 712 (A). Δ752, Δ769, Δ784, Δ793, Δ800, Δ812, and Δ831 had stop codons inserted after amino acids 752, 769, 784, 793, 800, 812, and 831, respectively (B). The Stat2 proteins were precipitated from these cell lines with Stat2n antibody, and the blots were first probed with anti-phosphotyrosine antibody (4G10) to locate the tyrosine-phosphorylated proteins (P-Tyr Blot) and then with Stat2n antibodies (Stat2 Blot).

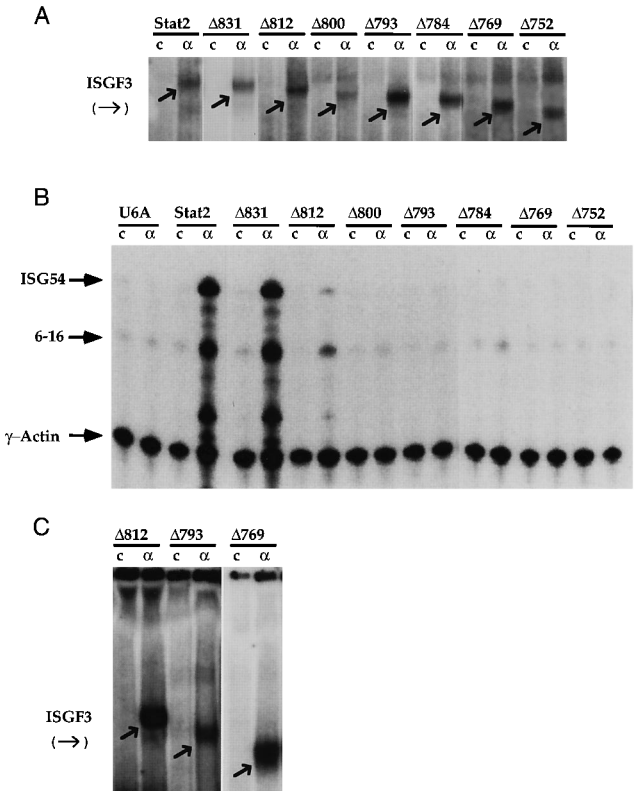

FIG. 6. IFN-α induction of the ISGF3 activity and the ISG54 and 6-16 RNAs in U6A cell derivatives. Cell lines expressing Stat2 carboxy-terminal deletion mutations were treated (α lanes) with IFN-α for 45 min before a DNA binding assay (EMSA) or for 4 h before RNA analysis or were left untreated (c lanes). (A) ISGF3 activity was determined by EMSA with a ^{32}P-labeled ISRE from the ISG15 gene as a probe and whole-cell extracts. Arrows indicate the positions of ISGF3. (B) RNA levels of 6-16, ISG54, and γ-actin were determined by an RNase protection assay (see Materials and Methods). (C) Nuclear translocation of ISGF3. ISGF3 activity in nuclear extracts (with equal amounts of total proteins) was determined with cell lines expressing Stat2 with stop codons after amino acid 812 (Δ812), 793 (Δ793), and 769 (Δ769). Arrows indicate the positions of ISGF3.

interpretation. The -SH2 mutant (R-601→K-601) cannot attach to the receptor and therefore has no effect on the phosphorylation of the endogenous wild-type protein, but the Y-690→F-690 mutant may attach to the receptor-kinase complex, be unable to become phosphorylated, and therefore remain associated with the receptor for a longer than normal time, thus impeding access of wild-type protein to the site. The same is possible for the ΔTY mutant. Because a complete block of IFN-α activation was not observed, it seems likely that the Y-690→F and ΔTY mutants do eventually disengage from the receptor-kinase complex, allowing access of the wild-type protein at a reduced frequency.

Three other mutations in the region around the Y-690 residue were examined in permanently transfected cells. The R-687–R-688–K-689 sequence (just upstream from Y-690) was changed to N-N-M. This change had no effect on Stat2 and Stat1 activation, ISGF3 formation, nuclear translocation, or IFN-α transcriptional response (Fig. 4 [NNM lanes]) (data not shown). Similar results were obtained when K-692 was changed to a methionine (Fig. 4 [M692 lanes]). Mutation of R-694 to N resulted in a mild decrease in phosphorylation after IFN-α treatment of Stat2 and also a decrease in phosphorylation of Stat1.

Effect of amino- and carboxyl-terminal deletions. We next turned to deletion mutations of the termini of the Stat2 molecule to determine whether any expendable sequences could be found. Deletion of as few as five amino acids from the amino terminus of the molecule had a distinct effect on the extent of phosphorylation at the remote Y-690 residue. Deletion of 25 amino acids decreased tyrosine phosphorylation by more than 90%, and deletion of the amino-terminal 59 amino acids completely prevented tyrosine phosphorylation (Fig. 5A). The inability to become phosphorylated was correlated with the inability to support Stat1 phosphorylation (Fig. 5A [Stat1 band in Δ25 and Δ59 lanes]), giving further indication that tyrosine phosphorylation of Stat2 appears to be required for the tyrosine phosphorylation of Stat1 at the IFN-α receptor.

With regard to deletions of the carboxyl terminus, residues were removed to positions 831, 812, 800, 793, 784, 769, 752, and 712, and expression constructs of these deletions were prepared and introduced into U6A cells, which were then tested for several IFN-α responses. The shortened proteins, including deletion 712, were all phosphorylated on tyrosine (Fig. 5). (The phosphorylation of Δ752 separate from Stat1 was not possible to distinguish, since the putative Stat1 and the truncated Stat2 comigrated, but Δ752 did form ISGF3 [see below].) All -COOH truncations which themselves were phosphorylated on tyrosine were also capable of supporting Stat1 tyrosine phosphorylation in response to IFN-α (Fig. 5).

All carboxyl-terminal deletions were capable of participating in successively faster-migrating, IFN-α-induced DNA protein complexes with the ISRE (Fig. 6A). However, these truncations uncovered an important functional role for the carboxyl

TABLE 1. Effects of Stat2 mutations on IFN-α-induced signaling[a]

Stat2 protein	Type of activity							
	Tyrosine-phosphorylated Stat1	Tyrosine-phosphorylated Stat2	ISGF3[b]	AAF[b]	Stat1-Stat2 heterodimer	ISG54[c]	6-16[c]	IRF-1[c]
None[d]	−	−	−	−	−	−	−	−
Wild type	+	+	+	+	+	+	+	+
Δ5	+/−[e]	−	−	−	−	ND	ND	ND
Δ15	−[e]	−	−	−	−	ND	ND	ND
Δ25	−	−	−	−	−	ND	ND	ND
Δ59	−	−	−	−	−	ND	ND	ND
Δ831	+	+	+	+	+	+	+	+
Δ812	+	+	+	+	+	+/−	+/−	+/−
Δ800	+	+	+	+	+	−	−	−
Δ784	+	+	+	+	+	−	−	−
Δ767	+	+	+	+	+	−	−	−
Δ752	+	+	+	+	+	−	−	−
Δ712	+	+	+	+	+	−	−	−
K538[f]	+	+	+	+	+	ND	ND	ND
K601	−	−	−	−	−	ND	ND	ND
F690	−	−	−	−	−	ND	ND	ND
NMM	+	+	+	+	+	+	+	+
M692	+	+	+	+	+	ND	ND	ND
N694[g]	+/−	+/−	+/−	+/−	+	ND	ND	ND
V1,4,5[h]	+	+	+	+	+	+	+	ND
V2,4,5[h]	+	+	+	+	+	+	+	ND
V1,2,4,5[h]	+	+	+	+	+	+	+	+

[a] Cell lines expressing various Stat2 mutants in U6A cells were assayed (as described in Materials and Methods) for various aspects of IFN-α-induced signaling. +, full activity; +/−, partial activity; −, no detectable activity; ND, not determined.

[b] ISGF3 and IFN-α activation factor (AAF) activities were determined by EMSA, with either an ISRE from the ISG15 gene or a GAS from either the LyE6 or the GRR gene being used.

[c] IFN-α-induced gene expression for these genes was examined by an RNase protection assay (13).

[d] Data are from U6A cells which lack Stat2 (13).

[e] A low level of phosphorylation of Stat2 is observed in cells expressing these deletions.

[f] Stat2 with Trp-538 changed to lysine in the SH3 domain.

[g] This mutation resulted in decreased Stat1 and Stat2 phosphorylation.

[h] Stat2 with leucine (L)-to-valine (V) mutations as indicated in Fig. 1. These mutations correspond to Leu-198 (V1), Leu-206 (V2), Leu-220 (V4), and Leu-227 (V5).

terminus. When IFN-α induction of mRNAs from genes (ISG54 and 6-16) earlier shown to be transcriptionally induced by IFN-α (3, 12) was tested, it was clear that transcriptional activation required amino acids at the carboxyl terminus. A deletion from the full-length 851 amino acids of Stat2 to 831 amino acids had little effect on transcriptional activation, but cells expressing a protein with a deletion to residue 812 gave only a small IFN-α-dependent transcriptional response (Fig. 6B [Δ812]). Deletion to residue 800 or beyond completely killed all induced transcriptional activity (Fig. 6B). All of the transcriptionally inactive proteins produced transcriptional complexes competent to form ISGF3 and move to the nucleus (Fig. 6C) (data not shown). Thus, the carboxyl terminus must function in transcriptional activation and not in some other event required in the JAK-STAT pathway.

DISCUSSION

In this paper, we have described the effects of various mutations introduced into Stat2 to understand its role in IFN-α signaling. The results, obtained with a number of Stat2 mutants, are summarized in Table 1.

There are two central results that come from these experiments, aside from validation of the importance of tyrosine 690, the only known tyrosine phosphorylation site in the molecule, and proof of a crucial role for the SH2 group, illustrated by the lack of tyrosine phosphorylation of R-601→K-601. This mutation in the pocket of the -SH2 group (31) would prevent Stat2 from interacting with phosphotyrosine on either the JAK kinase or the IFN-α receptor chains. A major unresolved issue to which the present experiments are addressed are the events at the IFN-α receptor leading to phosphorylation of Stat2 and Stat1. As was outlined in the original description of U6A cells (13), the complete absence of the Stat2 protein is associated with a failure of phosphorylation of Stat1 in IFN-α-treated cells. In the present work, we show that any of the mutations tested which causes lack of phosphorylation of Stat2 leads to failure of IFN-α to induce tyrosine phosphorylation of Stat1. These mutations include not only R-601→K-601 in the SH2 domain but also amino-terminal truncations which, for unknown reasons, block Stat2 tyrosine phosphorylation at the distant Y-690 site. Thus, we are led to the conclusion that either the Stat2 tyrosine phosphate itself is required as a docking site for Stat1 or the formation of a receptor-kinase complex competent to attract and phosphorylate Stat1 requires phosphorylated Stat2. For example, it might be that phosphorylated Stat2 attracts an additional transmembrane receptor chain or allows the JAK kinases access to a receptor tyrosine site that binds Stat1 after phosphorylation. What is strongly indicated is that a phosphorylated Stat2 and not simply an immobilized nonphosphorylated Stat2 is required for Stat1 phosphorylation. For example, disabled Stat2 mutants that cannot become phosphorylated and that might be expected to clutter the receptor-kinase complex (Y→F-690 and ΔTY) do in fact suppress the activation of endogenous Stat2, probably by binding to the IFN-α receptor. However, they do not lead to phosphorylation of Stat1, so it appears that for Stat2 to promote Stat1 phosphorylation, Stat2 itself must be phosphorylated.

However, even if a Stat1 molecule is recruited to the IFN-α receptor through the action of a phosphorylated Stat2, it does not obligatorily associate with that Stat2 molecule. We know that both Stat1-Stat2 and Stat1-Stat1 dimers form after IFN-α treatment (20). At the moment, too few details are settled to propose a specific model for dimer formation. For example, as mentioned above, even the number of receptor chains in the functional IFN-α receptor is not known, and which chains are phosphorylated is also unknown. It is known that the IFN-γ receptor chain (6) and the gp130 chain of other cytokine receptors contain tyrosines that become phosphorylated after ligand binding and that these phosphotyrosines are the likely docking sites for Stat1 and Stat3. It is not yet known in any case whether the possible members of a STAT dimer pair come from the same or two different receptor chains, and it is not known whether the two molecules of a dimer ever exist free in solution or dimerize during exit from the receptor. In vitro experiments in which an assay for direct attachment to an activated receptor-kinase complex of nonphosphorylated STATs and detachment of phosphorylated (monomeric or dimeric) protein is carried out would certainly help in further understanding of this situation, but such experiments with broken-cell preparations have not yet succeeded.

The second major issue to which the present results apply is the crucial transcriptional activation role in ISGF3 for Stat2. Stat1 can function in ISGF3 either with or without the COOH-terminal 38 residues known to be required for Stat1 gene activation (17). This is not the case for the COOH-terminal residues of Stat2. Deletion of 50 amino acids of the 851-residue Stat2 molecule renders ISGF3 containing this protein unable

to stimulate transcription. The COOH terminus of Stat1 has a required serine residue for IFN-γ induction in a sequence that is conserved between Stat1, Stat3, Stat4, and Stat5 (33). In contrast, Stat2 has a different -COOH terminus featuring a large concentration of acidic amino acids. However, deletion to residue 800, which inactivates the IFN-α transcriptional response, removes 7 but leaves 24 acidic residues in a 100-amino-acid stretch. Therefore, the simple provision of acidic residues would appear to be insufficient to activate transcription. These results show that some (Stat1-Stat1, for example) but not all (ISGF3) STAT-containing transcription factors may be affected by serine phosphorylation. Finally, it should be noted that p48 is also part of ISGF3 and could also contribute to transcriptional activation. Analysis of that protein is under way in order to determine its contribution to transcriptional activation.

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