MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 2048–2056 Vol. 17, No. 4
0270-7306/97/$04.00+0
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Functional Subdomains of STAT2 Required for Preassociation with the Alpha Interferon Receptor and for Signaling

XIAOXIA LI,[1] STEWART LEUNG,[1] IAN M. KERR,[2] AND GEORGE R. STARK[1]*

Department of Molecular Biology, Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195,[1] and Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom[2]

Received 22 July 1996/Returned for modification 29 August 1996/Accepted 3 January 1997

Two members of the STAT signal transducer and activator of transcription family, STAT1 and STAT2, are rapidly phosphorylated on tyrosine in response to alpha interferon (IFN-α). Previous work showed that in the mutant human cell line U6A, which lacks STAT2 and is completely defective in IFN-α signaling, the phosphorylation of STAT1 is very weak, revealing that activation of STAT1 depends on STAT2. We now find that STAT2 binds to the cytoplasmic domain of the IFNAR2c (also known as IFNAR2-2) subunit of the IFN-α receptor in extracts of untreated cells. STAT1 also binds but only when STAT2 is present. The activities of chimeric STAT2-STAT1 proteins were assayed in U6A cells to define regions required for IFN-α signaling. Previous work showed that a point mutation in the Src homology 2 (SH2) domain prevents STAT2 from binding to phosphotyrosine 466 of the IFNAR1 subunit of the activated receptor. However, we now find that the entire SH2 domain of STAT2 can be replaced by that of STAT1 without loss of function, revealing that other regions of STAT2 are required for its specific interaction with the receptor. A chimeric protein, in which the N-terminal third of STAT2 has replaced the corresponding region of STAT1, did preassociate with the IFNAR2c subunit of the receptor, became phosphorylated when IFN-α was added, and supported the phosphorylation of endogenous STAT1. These results are consistent with a model in which STAT2 and STAT1 are prebound to the IFNAR2c subunit of the resting receptor. Upon activation, the IFNAR1 subunit is phosphorylated on Tyr-466, allowing the SH2 domain of STAT2 to bind to it; this is followed by the sequential phosphorylation of STAT2 and STAT1.

Many cytokines and growth factors utilize proteins of the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) families to propagate extracellular signals (reviewed in references 4, 16, 30). Upon ligand binding, the STATs are activated by phosphorylation on tyrosine and released as hetero- or homodimers which migrate to the nucleus where they activate the transcription of specific genes. In response to alpha interferon (IFN-α), STAT1-STAT2 heterodimers combine with the DNA-binding protein p48 to form IFN-stimulated gene factor 3 (ISGF3), which binds to the IFN-stimulated response elements (ISREs) of many IFN-α-responsive genes.

All STATs contain Src homology 2 (SH2) domains, which are vital for function. The mechanism of STAT activation is fairly well understood in the IFN-γ signaling pathway, where only STAT1 is activated. Upon IFN-γ binding, the α subunit of the receptor is rapidly phosphorylated on Tyr-440, which then binds to the SH2 domain of STAT1 (10), allowing it to be phosphorylated on Tyr-701 by JAK1 or JAK2, which are both required for IFN-γ signaling (24, 35). Reciprocal SH2-phosphotyrosine binding is also required to form STAT1 homodimers, which activate transcription through gamma-activated sequence (GAS) elements (31). Similar SH2-mediated STAT activation mechanisms have been proposed for many other cytokine and growth factor signaling pathways (3, 9, 15, 33).

Studies of mutant human cell lines lacking specific components required for signaling have provided extensive information on the mechanism of STAT activation in response to IFN-α (4, 26). The mutant cell line U6A lacks STAT2 and is completely defective in response to IFN-α (20). The defect is complemented by wild-type STAT2 but not by variants with mutations in the SH2 domain or Tyr-690, the site of phosphorylation (28). Furthermore, STAT1 phosphorylation in response to IFN-α is greatly reduced in U6A cells, in contrast to the normal phosphorylation of STAT2 in U3A cells (17), which lack STAT1 (23). These results, together with knowledge of the mechanism of IFN-γ-mediated STAT1 activation, led to a sequential activation model for IFN-α signaling (20). In response to IFN-α, STAT2 docks to a phosphotyrosine in the activated IFN-α receptor and is phosphorylated on Tyr-690 (11), which then provides a docking site for the SH2 domain of STAT1, allowing its Tyr-701 to become phosphorylated. The IFN-α receptor has two functional subunits, IFNAR1 (34) and IFNAR2c (5, 22). Recently, the docking site for STAT2 has been shown to be Tyr-466 of the IFNAR1 subunit of the receptor (37). IFNAR2c, the protein product of an alternatively spliced form of the IFNAR2 gene, has been shown to complement the defect in mutant U5A cells, proving that it plays a crucial role in signaling (22).

The specific recognition of receptor phosphotyrosine residues by STAT SH2 domains is likely to play an important role in determining which STATs are activated in different signaling pathways (13). For example, when the SH2 domain of STAT2 is replaced by that of STAT1, the chimeric protein is activated by IFN-γ in U3A cells, which lack STAT1. However, when the SH2 domain of STAT1 is replaced by that of STAT2, the chimeric protein is not activated by IFN-α in U6A cells, which lack STAT2, suggesting that the SH2 domain of STAT2, although necessary for activation of STAT2 in response to IFN-α, is not sufficient (13). Furthermore, Qureshi et al. (28) demonstrated that deletion of 59 amino acids from the N-terminal domain of STAT2 inactivated its ability to become phosphorylated in response to IFN-α, revealing that regions of

* Corresponding author. Mailing address: Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195.

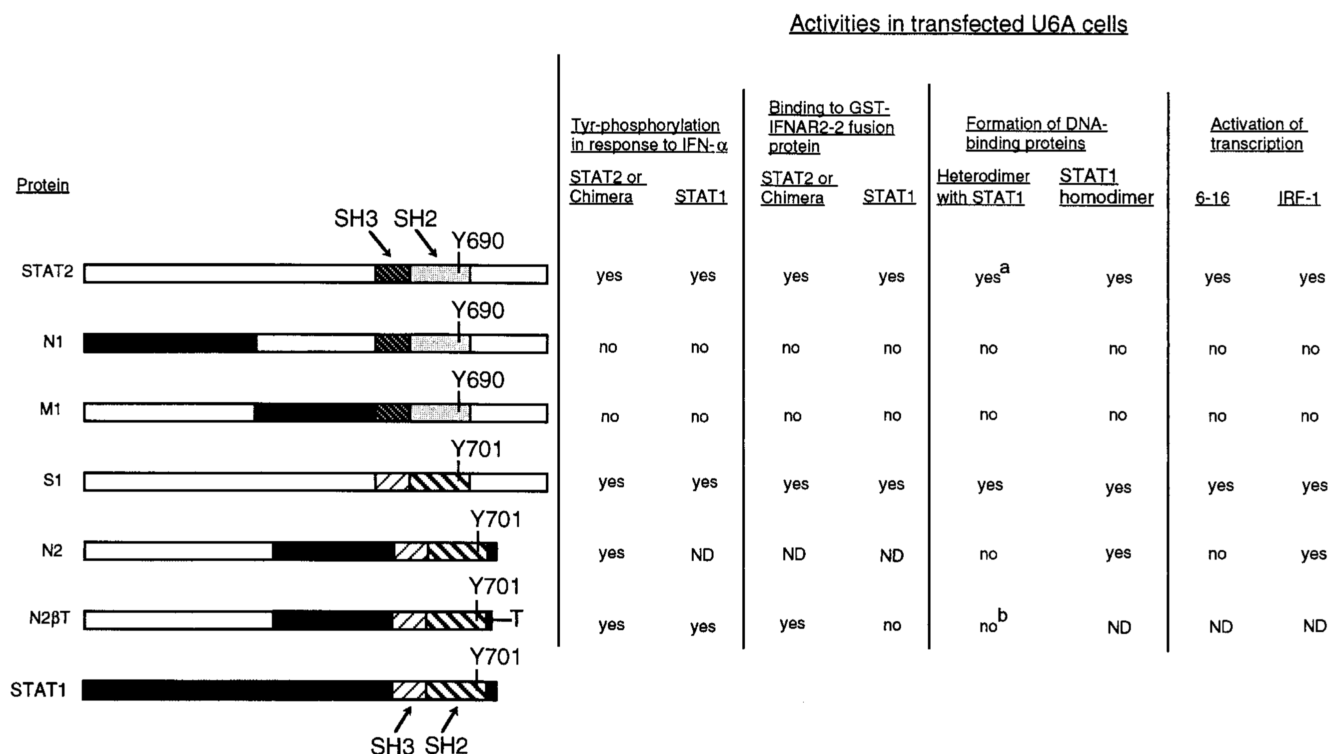


FIG. 1. Summary of the structures and activities of STAT1-STAT2 chimeric proteins. The constructs are shown schematically, with STAT2 in white and STAT1 in black. The SH3 and SH2 domains and the conserved tyrosine phosphorylation sites of STAT2 (Y690) and STAT1 (Y701) are also depicted. ND, not determined; a and b, see reference 21.

STAT other than the SH2 domain provide essential interactions with other signaling components. To refine and extend these studies, we have exchanged additional regions of STAT1 and STAT2 and have assayed the functions of the resulting chimeric proteins in U6A cells. Preassociation of STAT2 with the IFNAR2c subunit of the receptor depends on the N-terminal third of STAT2 and is crucial both for subsequent STAT2 activation in response to IFN-α and for the STAT2-dependent activation of STAT1.

MATERIALS AND METHODS

Cells and IFNs. 2fTGH, U6A, and U3A cells were grown as described previously (26). Human recombinant IFN-α2 (3.6×10^5 IU/mg, or 5×10^6 IU/ml) from Hoffmann LaRoche was used at a final concentration of 500 IU/ml.

Constructs. Chimeric cDNAs, constructed by the gene splicing by overlap extension technique (14), were subcloned into the eukaryotic expression vector pMNC (27). In the constructs, many of which are depicted schematically in Fig. 1, the amino acid (aa) numberings are according to the numbering scheme of Fu et al. (8). For construct N1 aa 1 to 294 of STAT2 were replaced by aa 1 to 284 of STAT1. For construct N1T a FLAG octapeptide epitope tag (Kodak) was added to the C terminus of N1. For construct M1 aa 295 to 514 of STAT2 were replaced by aa 285 to 504 of STAT1. For construct S1 aa 515 to 700 of STAT2 were replaced by aa 505 to 701 of STAT1. For construct N2 aa 1 to 305 of STAT1α were replaced by aa 1 to 315 of STAT2. For construct N2βT aa 1 to 305 of STAT1β were replaced by aa 1 to 315 of STAT2 and a FLAG epitope tag was added to the C terminus. To express it as a glutathione *S*-transferase (GST)-fusion protein, most of the cytoplasmic domain of the IFNAR2c cDNA (5, 22), aa 244 to 462, was subcloned into pGEX2T (Pharmacia). The full-length cytoplasmic domain extends from aa 244 to 515.

Peptide binding assays. Biotin-labeled phosphopeptides spanning Tyr-466 (RCINY[PO_4]VFFPSLKPS), Tyr-481 (SIDEY[PO_4]FSEQPLKNL), and Tyr-527 (DHKKY[PO_4]SSQTSQDSG) of IFNAR1 (34) and the Tyr-466 peptide without phosphate were synthesized (10). The Tyr-466 peptides with or without phosphate were dissolved in glacial acetic acid, and the other peptides were dissolved in water. Preparation of U6A-STAT2 cell extracts and peptide binding experiments were performed as described by Greenlund et al. (10), except that the peptides were bound to streptavidin beads first.

Immunoprecipitations. Tyrosine phosphorylation of STATs was monitored as described by Schindler et al. (29). Antibodies against the N-terminal or C-terminal region of STAT2 (8, 27) were used to immunoprecipitate it and appropriate chimeric proteins. The anti-STAT1 used was described previously (20, 27). Anti-FLAG M2 antibody was from Kodak. Tyrosine phosphorylation was detected by using monoclonal antibody PY20 (Transduction Laboratories).

Experiments with the GST-fusion protein. The GST-IFNAR2c fusion protein, expressed in *Escherichia coli* SURE, was prepared as described by Frangioni and Neel (7); precipitations were performed as described by Kaelin et al. (18). Briefly, $\sim 10^7$ cells, washed with ice-cold phosphate-buffered saline, were pelleted and lysed in 200 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 25 mM HEPES [pH 7.5], 1 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride) for 30 min on ice. The lysates were cleared by spinning at $16,000 \times g$ for 10 min. About 1 μg of GST-fusion protein coupled to 10 μl of glutathione beads was added to 100 to 200 μl of lysate and gently mixed for 4 to 5 h at 4°C. The beads were washed five times with ice-cold lysis buffer. Proteins bound to the beads were released by boiling in loading buffer and separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Cell lysates (about 10 μg of protein) were run in parallel to estimate the protein levels.

Other assays. Wild-type STAT2 and the chimeric constructs were transfected into U6A cells by the calcium phosphate method (20). Levels of the exogenous proteins were analyzed by Western blotting, and clones expressing similar levels were used. Specific complexes binding to either the inverted repeat (IR) element of the IRF-1 gene or the ISRE of the 9-27 gene were detected as described previously (21). Total RNA was prepared from IFN-α-treated cells, and RNase protection experiments were performed as described by Leung et al. (20). The probes used protect 190 bases of 6-16, 175 bases of IRF-1, and 130 bases of γ-actin RNA (20).

RESULTS

Role of the SH2 domain in the activation of STAT2. In the chimeric protein S1, the segment including the SH2, SH3, and tyrosine phosphorylation domains of STAT2 is replaced by the corresponding segment of STAT1 (Fig. 1). Surprisingly, in complemented U6A cells, S1 was phosphorylated on tyrosine in response to IFN-α (Fig. 2A, lanes 9 and 10) just as well as wild-type STAT2 (Fig. 2A, lanes 3 and 4), showing that, al-

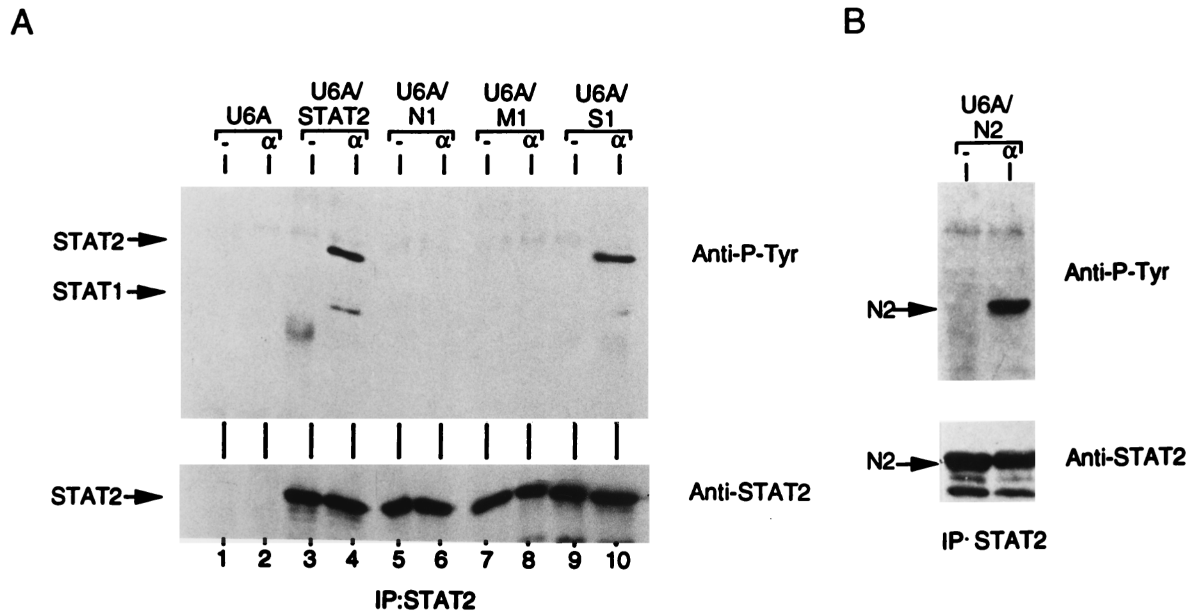


FIG. 2. IFN-α-mediated phosphorylation of STAT2 and chimeric proteins in U6A cells, untreated (−) or treated (α) with 500 IU of IFN-α per ml for 15 min. (A) Cell extracts were prepared from U6A cells (lanes 1 and 2) or U6A cells transfected with STAT2 (lanes 3 and 4), N1 (lanes 5 and 6), M1 (lanes 7 and 8), or S1 (lanes 9 and 10). Immunoprecipitation was performed with anti-STAT2 directed against the C-terminal region. Western transfers were probed first with anti-phosphotyrosine (top) and then with anti-STAT2 (bottom). (B) Same as for panel A except that the extracts were prepared from N2-transfected U6A cells. Immunoprecipitation was performed with anti-STAT2 directed against the N-terminal region. The Western transfer was probed first with anti-phosphotyrosine (top) and then with anti-STAT2 (bottom).

though the STAT2 SH2 domain is necessary for activation (28) it does not determine the specificity of this activation.

Role of the N-terminal region of STAT2. When the N-terminal or middle regions of STAT2 were replaced by the corresponding regions of STAT1 (Fig. 1, N1 and M1), the resulting chimeric proteins were no longer phosphorylated on tyrosine in response to IFN-α (Fig. 2A, N1 and M1), suggesting either that these two regions are required for specific activation of STAT2 or that the chimeric proteins do not assume a correctly folded structure. To investigate the role of the N-

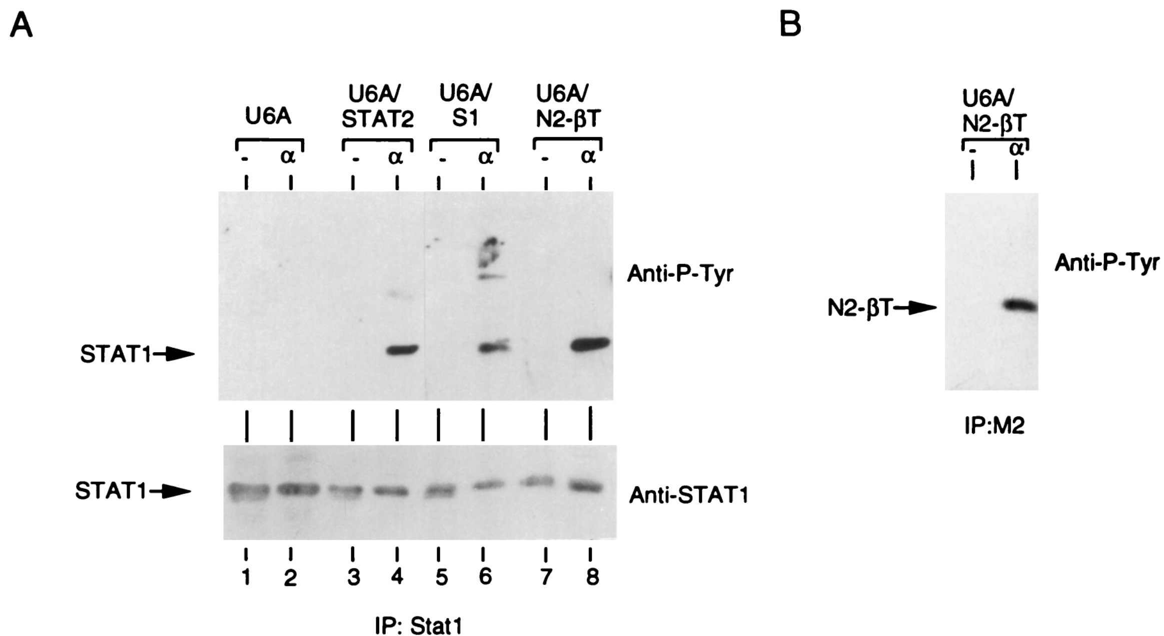


FIG. 3. (A) STAT1 tyrosine phosphorylation in U6A cells expressing STAT2, S1, or N2βT. The method used is the same as that described in the legend for Fig. 2. Immunoprecipitation was performed with anti-STAT1α directed against the C-terminal region. The Western transfer was probed first with anti-phosphotyrosine (top) and then with anti-STAT1α (bottom). (B) IFN-α-mediated tyrosine phosphorylation of N2βT. Extracts were prepared from N2βT-transfected U6A cells untreated (−) or treated with 500 IU of IFN-α per ml for 15 min. Immunoprecipitation was performed with anti-M2 epitope tag, and the Western transfer was probed with anti-phosphotyrosine.

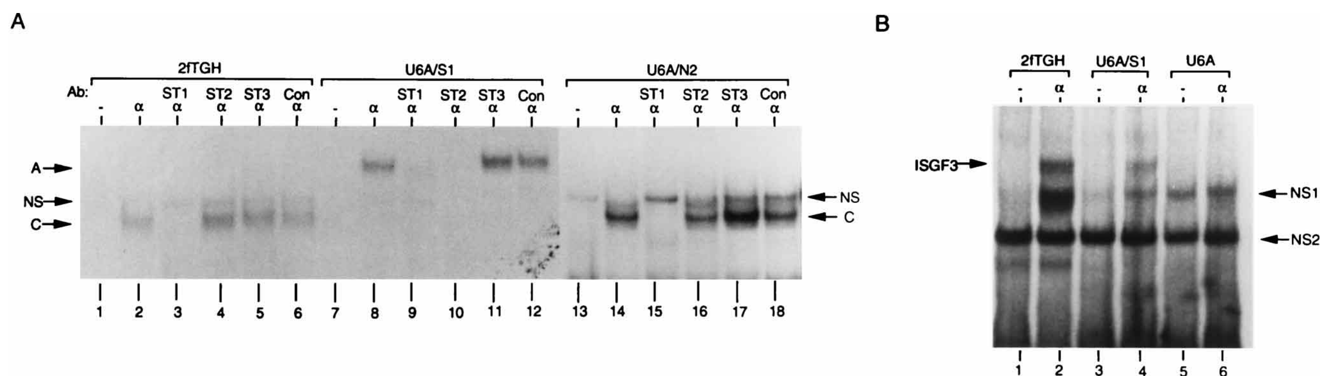
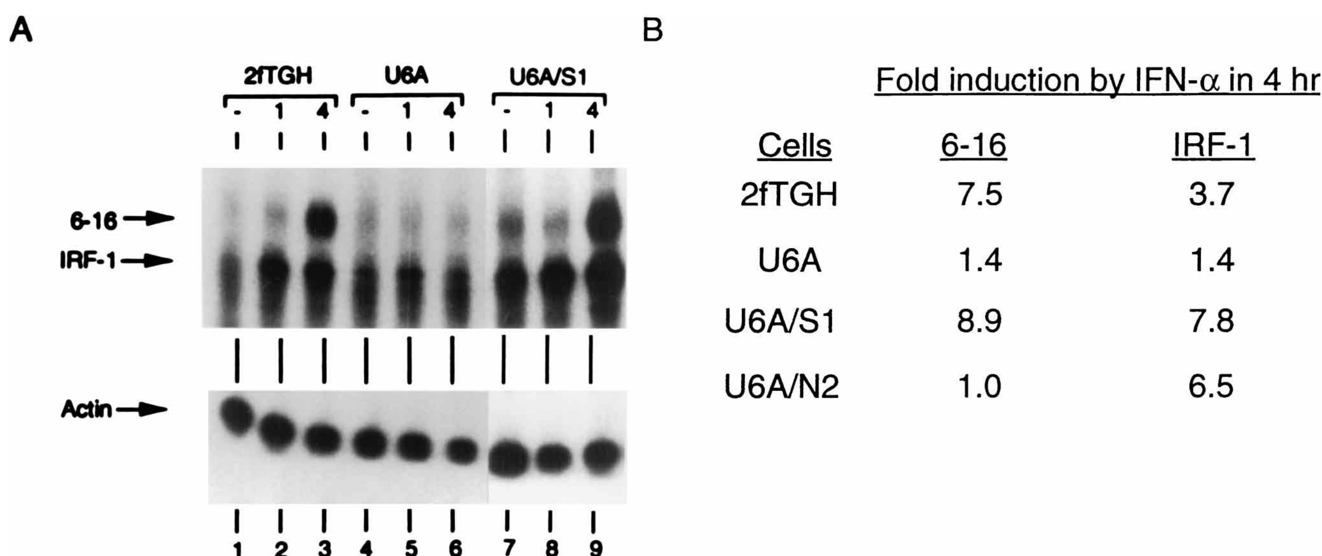


FIG. 4. Formation of band-shift complexes in IFN-α-treated U6A and control cells. (A) Extracts were prepared from 2fTGH (lanes 1 to 6), S1-transfected U6A (lanes 7 to 12), or N2-transfected U6A cells (lanes 13 to 18), untreated (−) or treated with 500 IU of IFN-α (α) per ml for 15 min. An IR probe from the IRF-1 gene was used. Antibodies against STAT1 (ST1, lanes 3, 9 and 15), STAT2 (ST2, lanes 4, 10 and 16), STAT3 (ST3, lanes 5, 11 and 17), or a control antibody (Con, lanes 6, 12 and 18) were used as indicated. The IFN-α-inducible complexes A and C and the constitutive nonspecific complex NS are indicated by arrows. Longer exposure of the autoradiogram revealed the formation of complex A in the 2fTGH extract, which corresponds to the previously described (21) STAT1-STAT2 heterodimer (data not shown). (B) Cells treated with 20 IU of IFN-γ per ml overnight were then left untreated (−) or treated (α) with 500 IU of IFN-α per ml for 15 min. Extracts were prepared from 2fTGH (lanes 1 and 2), S1-transfected U6A (lanes 3 and 4), and U6A (lanes 5 and 6) cells. A 9-27 ISRE probe was used. Two ISGF3 complexes were observed, probably containing either STAT1α or STATβ. The slower-migrating complex is indicated by an arrow. NS1, a constitutive nonspecific band (lanes 5 and 6); NS2, a nonspecific band.

terminal region further, the first 315 amino acids of STAT2 were swapped into STAT1 (Fig. 1, construct N2). N2 is strongly phosphorylated on tyrosine in response to IFN-α in U6A cells (Fig. 2B). Since the N-terminal region of STAT2 can transform STAT1 into a STAT2-like molecule, it must be important in mediating specific STAT2-receptor interactions. It was also found that N2 is phosphorylated in response to IFN-α in U3A cells, which lack STAT1 (data not shown).

Regions of STAT2 required for STAT1 activation. The phosphorylation of STAT1 is very weak when STAT2 is absent (20) (Fig. 3A, lanes 1 and 2). To identify subregions of STAT2 required to support tyrosine phosphorylation of native STAT1, IFN-α-treated U6A cells expressing different chimeric proteins were studied. Tyrosine phosphorylation of STAT1 was not observed in cells expressing N1 or M1 (data not shown), whereas S1 (Fig. 3A, lanes 5 and 6), like wild-type STAT2 (lanes 3 and 4), did support STAT1 phosphorylation. As noted above, the N2 construct is phosphorylated in U6A cells. The N2 chimeric protein is similar in size to endogenous STAT1, and it also reacts with the anti-STAT1α we have used, which is directed against the C terminus, making analysis of STAT1 phosphorylation difficult in U6A cells expressing N2. Therefore, we constructed N2βT, in which aa 1 to 305 of STAT1β are replaced by aa 1 to 315 of STAT2 and in which a FLAG epitope tag is present at the C terminus (Fig. 1). When immunoprecipitated by an anti-FLAG M2 antibody, N2βT (Fig. 3B), like N2 (Fig. 2B), was phosphorylated on tyrosine in response to IFN-α. N2βT did not react with the antibody to the C terminus of STAT1α, allowing us to immunoprecipitate STAT1α specifically. STAT1α was phosphorylated on tyrosine



Cells	6-16	IRF-1
2fTGH	7.5	3.7
U6A	1.4	1.4
U6A/S1	8.9	7.8
U6A/N2	1.0	6.5

FIG. 5. IFN-α-induced expression of the 6-16 and IRF-1 genes in 2fTGH, U6A, and U6A cells transfected with S1. (A) Total RNA, prepared from cells untreated (−) or treated with IFN-α for 1 h (1) or 4 h (4), was analyzed by RNase protection with probes for 6-16, IRF-1, and γ-actin. (B) Quantitation of the experiment. The intensities of the bands in untreated cells or in cells treated for 4 h were measured by using a PhosphorImager, and the signals were normalized to γ-actin. Induction levels relative to the untreated samples are presented. The data for U6A/N2 are from reference 21.

FIG. 6. In vitro binding of STAT2 and STAT1 to phosphopeptides of IFNAR1. A cell extract was prepared from STAT2-transfected U6A cells, and a portion was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lane 1). Other portions were incubated with biotin-labeled phosphopeptides spanning p-Tyr-466 (lane 2), p-Tyr-481 (lane 3), or p-Tyr-527 (lane 4) of IFNAR1, schematically shown at the top. The biotin-labeled phosphopeptides were bound to avidin-sepharose beads, and the released proteins were separated by electrophoresis. The Western transfer was probed first with anti-STAT2 (top) and then with anti-STAT1 (bottom).

in response to IFN-α in U6A cells expressing N2βT (Fig. 3A, lanes 7 and 8). The N2βT results suggest that the N-terminal region of STAT2 is important both for the phosphorylation of STAT2 and for the STAT2-dependent phosphorylation of STAT1.

Formation of STAT dimers in U6A cells transfected with chimeric proteins. Li et al. (21) found that STAT1-STAT2 heterodimers bind to the IR element of the IRF-1 gene in the absence of p48. Similarly, S1 forms a heterodimer with STAT1, which binds to an IR probe (Fig. 4A, lanes 7 to 12, complex A). STAT1 homodimers in extracts of IFN-α-treated, S1-transfected U6A cells were detected only after long exposure of the autoradiograms (data not shown), similar to our previous observations with STAT2-transfected U6A cells (21). Furthermore, S1 did participate in forming ISGF3-like factors, detected with a 9-27 ISRE probe (Fig. 4B, lanes 3 and 4), revealing that the SH2 domain of STAT2 can be replaced by that of STAT1 in band-shift assays. The 6-16 and IRF-1 genes, not activated by IFN-α in U6A cells (20) (Fig. 5A, lanes 4 to 6), are activated in IFN-α-treated U6A cells expressing S1 (Fig. 5A, lanes 7 to 9). Thus, S1 is functionally very similar to wild-type STAT2. We detected a complex which migrated like STAT1 homodimers with the IR probe and extracts of U6A cells transfected with N2 (Fig. 4A, lanes 13 to 18, complex C) or N2βT (data not shown). An amount of antibody against the N terminus of STAT2 sufficient to supershift all S1-STAT1 heterodimers (Fig. 4A, lane 10) had only a minimal effect on the complex formed in a U6A-N2 extract (Fig. 4A, lane 16). Furthermore, N2βT did not coprecipitate with STAT1 (Fig. 3A, lanes 7 and 8, Fig. 3B, and data not shown). These observations indicate that although N2 can be activated similarly to STAT2 and can support STAT1 activation, it does not form homodimers or heterodimers with STAT1. However, since N2

FIG. 7. (A) Dependence of STAT binding on phosphorylation of Tyr-466. The experiment is the same as the one shown in Fig. 3, except that the p-Tyr-466 peptide (lane 2) was compared with the same peptide without phosphate (lane 3). The same experiment was performed with extracts of U6A cells (B) or of U6A cells expressing the S1 chimeric protein (C).

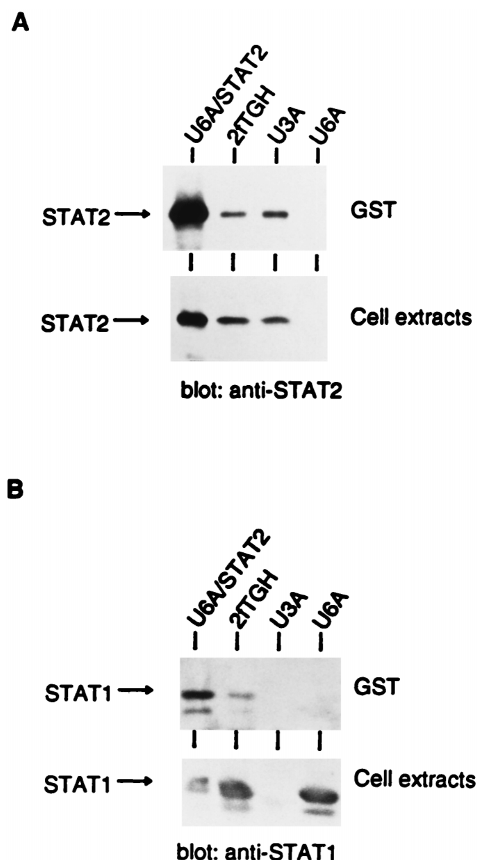


FIG. 8. Stabilization by STAT2 of STAT1-IFNAR2c association. (A) Proteins bound to the GST-IFNAR2c fusion protein were revealed by electrophoresis in denaturing polyacrylamide gels (top). Total cell extracts were run in the same gel (bottom). The extracts used are indicated at the top of the gel. The Western blot was probed with an anti-STAT2 monoclonal antibody. (B) The same experiment, except that the blot was probed with anti-STAT1 polyclonal antibodies.

does promote the formation of STAT1 homodimers (21), the IRF-1 gene is induced by IFN-α in N2-transfected U6A cells.

Identification of the STAT docking site of IFNAR1. The IFNAR1 subunit (34) is rapidly phosphorylated on Tyr-466 in response to IFN-α, the tyrosine residue most proximal to the transmembrane domain (1, 3). We studied three biotin-labeled phosphopeptides representing Tyr-466, Tyr-481, or Tyr-527 of the cytoplasmic domain of IFNAR1. In an in vitro assay, the phosphorylated Tyr-466 peptide (p-Tyr-466) bound to STAT2 (Fig. 6, top of lane 2) and also to STAT1 (Fig. 6, bottom of lane 2) (the intensity of the STAT1 band is weak in this experiment, but the intensity is stronger in other experiments, as for example, in Fig. 7A). The p-Tyr-481 and p-Tyr-527 peptides did not bind to either STAT (Fig. 6, lanes 3 and 4). p-Tyr-466 also bound to STAT3, but poorly (Fig. 7A, bottom of lane 2). The control unphosphorylated Tyr-446 peptide bound to STAT2 very poorly (Fig. 7A, compare lanes 2 and 3). Thus, p-Tyr-466 can be recognized by both STAT2 and STAT1. To determine whether STAT1 binds to p-Tyr-466 directly or through STAT2, we used extracts of U6A cells and found that STAT1 still binds to p-Tyr-466, showing that this binding is independent of STAT2 (Fig. 7B). The S1 chimeric protein also bound to p-Tyr-466, albeit weakly (Fig. 7C). The fact that the p-Tyr-466 docking site of IFNAR1 can be recognized by both STATs suggests that direct interaction between this residue and the SH2 domains of both STAT1 and STAT2, although essential, is not sufficient to establish specific, sequential activation of these STATs in response to IFN-α.

Cooperative association of STAT1 and STAT2 with the IFNAR2c subunit of the IFN-α receptor. We investigated possible interactions between the recently cloned IFN-α receptor subunit IFNAR2c (5, 22) to STATs. A GST-fusion protein containing 219 aa (aa 244 to 462) of the cytoplasmic domain of IFNAR2c binds to STAT1 and STAT2 in vitro (Fig. 8). Since IFN-α treatment is not necessary for binding, these two STATs are likely to preassociate with IFNAR2c. This experiment does not address the issue of whether other proteins are required or involved in receptor-STAT interactions. In U3A extracts (lacking STAT1) the binding of STAT2 to IFNAR2c was normal (Fig. 8A), but there was fivefold less binding of STAT1 to IFNAR2c in U6A extracts (lacking STAT2) compared to 2fTGH extracts (Fig. 8B), even though the amounts of STAT1 in the two extracts were similar (Fig. 8B, bottom). The binding of both STAT2 and STAT1 was enhanced in extracts of cells overexpressing STAT2 (Fig. 8). These observations suggest that IFNAR2c, STAT2, and STAT1 preassociate in the cell and that STAT2 stabilizes the binding of STAT1. Furthermore, the preassociation is specific for STAT2 and STAT1, since STAT3 and STAT5 did not bind to the fusion protein (data not shown). In vitro experiments (2) with a truncated IFNAR2c fusion protein (aa 265 to 462) and the full-length cytoplasmic domain (aa 265 to 515) reveal that both bind to STAT1 and STAT2.

Association of STAT1-STAT2 chimeric proteins with IFNAR2c in vitro. Compared to STAT2, S1 binds weakly to the IFNAR2c GST-fusion protein (Fig. 9A). The lower affinity of S1 for IFNAR2c in vitro did not correlate with its strong tyrosine phosphorylation (Fig. 1A, lanes 9 and 10) and transcriptional activation (Fig. 5A, lanes 7 to 9) in vivo. The reason for this difference is not clear. It is possible that the SH2 domain of STAT2 may make S1 more susceptible to denaturation in our in vitro binding assay. N2βT also bound to our IFNAR2c fusion protein (Fig. 9A) and N1 or M1 did not bind (data not shown). Taken together, the results suggest that the N-terminal third of STAT2 contains regions important for binding to IFNAR2c. Furthermore, there is an excellent correlation between prebinding to IFNAR2c and IFN-α-induced phosphorylation of each chimeric protein (Fig. 1), suggesting that prebinding is likely to be a key step in determining the specificity of STAT2 activation in IFN-α signaling. Among the chimeric proteins, only S1 supports the binding of STAT1 to IFNAR2c (Fig. 9B, and data not shown). Interestingly, although N2βT did support STAT1 phosphorylation, it failed to support STAT1 binding to IFNAR2c (Fig. 9B), suggesting that preassociation of STAT1 with the receptor is not absolutely required for activation. This result also suggests that the first 315 residues of STAT2 are not sufficient to recruit STAT1 to the IFNAR2c preassociation complex.

DISCUSSION

Upon binding to specific cell surface receptors, many different cytokines and growth factors activate members of the JAK and STAT families. Some recent studies have addressed how specificity is achieved in transmitting signals from the receptors and kinases to the STATs. Since JAKs can phosphorylate STATs nonspecifically, it is unlikely that the JAK-STAT interaction confers the needed specificity of STAT activation. The activated α subunit of the IFN-γ receptor has a critical phosphotyrosine residue which binds to the SH2 domain of STAT1 (10). Specific activation of STAT3 by ciliary neurotrophic fac-

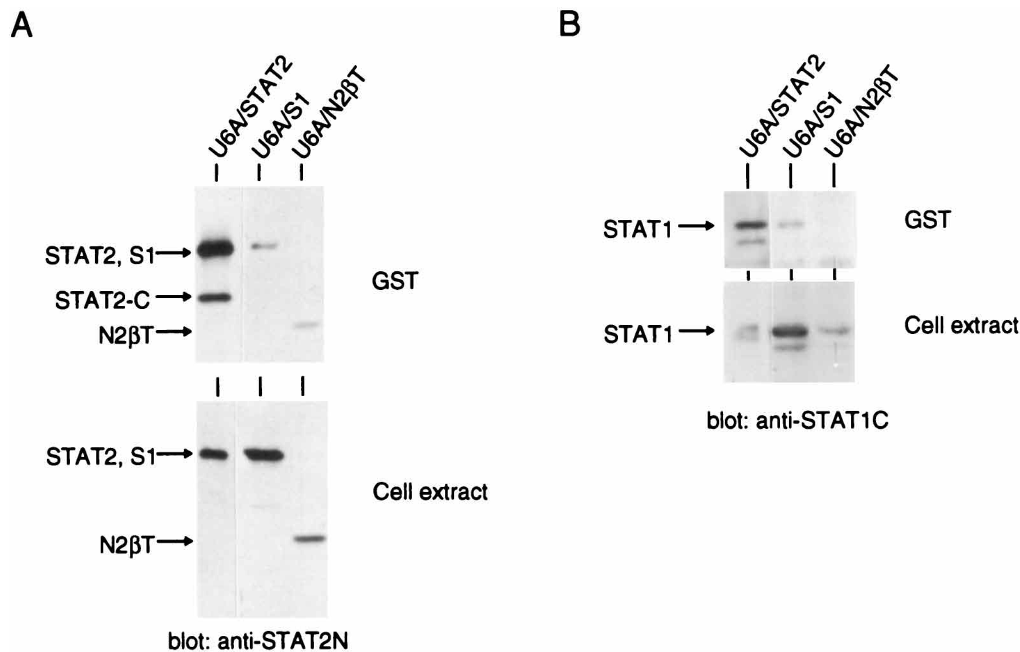


FIG. 9. Association of S1 and N2βT with GST-IFNAR2c. Proteins bound to the GST-IFNAR2c fusion protein were revealed by denaturing polyacrylamide gel electrophoresis (top). Extracts of untreated cells were run in the same gel (bottom). The extracts used are indicated on top of each gel. Analyses of Western transfers were performed with anti-STAT2 N terminus polyclonal (A) or anti-STAT1 C terminus polyclonal (B) antibodies. STAT2-C is a degradation product lacking part of the C-terminal region.

tor or interleukin 6 is determined by specific phosphotyrosine residues of gp130 or the leukemia inhibitory factor receptor (33). Based on studies such as these, it is generally believed that specific activation is determined by specific STAT SH2 domain-receptor phosphotyrosine interactions. However, we have now shown that an interaction involving the N-terminal domain plays a major role in determining the specific interaction of STAT2 with the IFN-α receptor.

Our previous sequential activation model (20) proposed that STAT2 plays a pivotal role in IFN-α signaling. It is recruited to the activated receptor first and, after phosphorylation by JAK kinases on tyrosine 690, provides a docking site for the SH2 domain of STAT1. To define how STAT2 is distinguished from STAT1 in IFN-α signaling, we generated a set of chimeric proteins and tested their ability to be activated in response to IFN-α. The most specific interaction required for activation is not recognition of a receptor p-Tyr residue by the STAT2 SH2 domain, since this domain can be replaced by that of STAT1. Therefore, although necessary, the STAT2 SH2 domain does not determine the specificity of the STAT2-receptor interaction. The N terminus of STAT2 converted STAT1 into a partial STAT2 mimic, revealing that the N-terminal third of the STAT2 protein contributes greatly to the specificity of its activation. These conclusions are supported by the results of Heim et al. (13), who found that a STAT1 chimeric protein containing the STAT2 SH2 domain was not activated by IFN-α in U6A cells, almost certainly because the protein lacks the essential N terminus of STAT2.

The activation of STAT2 is mediated in part by its preassociation with the IFN-α receptor IFNAR2c subunit. We found that STATs 1 and 2 associate with IFNAR2c in an extract of untreated 2fTGH cells. STAT2 binds efficiently without STAT1, but STAT1 does not bind as well when STAT2 is absent. Thus, STAT2 stabilizes the complex. Importantly, there was a correlation between binding to IFNAR2c and the IFN-α-induced phosphorylation of chimeric proteins, suggesting that preassociation with IFNAR2c is likely to be a key step in STAT2 activation in response to IFN-α. Interestingly, the chimeric protein N2βT failed to support STAT1 binding to IFNAR2c but still allowed STAT1 to be activated. Thus, preassociation of STAT1 to IFNAR2c is not absolutely required for activation or is too weak to register in the coprecipitation assay. Abramovich et al. (1) reported that STAT2, but not STAT1, associates with IFNAR1 in untreated cells, but we have not been able to confirm this association in our system.

The region of STAT2 that binds to IFNAR2c, known only at low resolution at present, corresponds to the N-terminal third of the molecule. Qureshi et al. (28) showed that IFN-α-induced phosphorylation of STAT2 was lost with an N-terminal deletion of only 59 amino acids, suggesting that the extreme N terminus of STAT2 is required for its phosphorylation.

A point mutation in the STAT2 SH2 domain abolishes its activity, indicating that SH2 function is indispensable (28). The p-Tyr-466 residue of IFNAR1 provides a docking site for the SH2 domain of STAT2. The p-Tyr-466 peptide can also bind to STAT1. This interchangeable binding to p-Tyr-466 supports the notion that docking of STAT1 and STAT2 to the receptor through their SH2 domains is not sufficient to determine the sequential activation of STAT2 and STAT1 by IFN-α. Yan et al. (37) expressed STATs in a baculovirus system and showed that p-Tyr-466 is specific for STAT2 but not for STAT1. This apparent discrepancy with our results might be explained by the different source of STATs or by the use of a peptide one amino acid shorter than the one we used.

Recently, two new functions have been attributed to the N-terminal region of STATs. Xu et al. (36) have found that the N termini of STAT1 and STAT4 mediate cooperative DNA binding of STAT homodimers to tandem GAS sites. Deletion of 44 residues from the N terminus of STAT1 abolished cooperative DNA binding. Shuai et al. (32) reported that the N

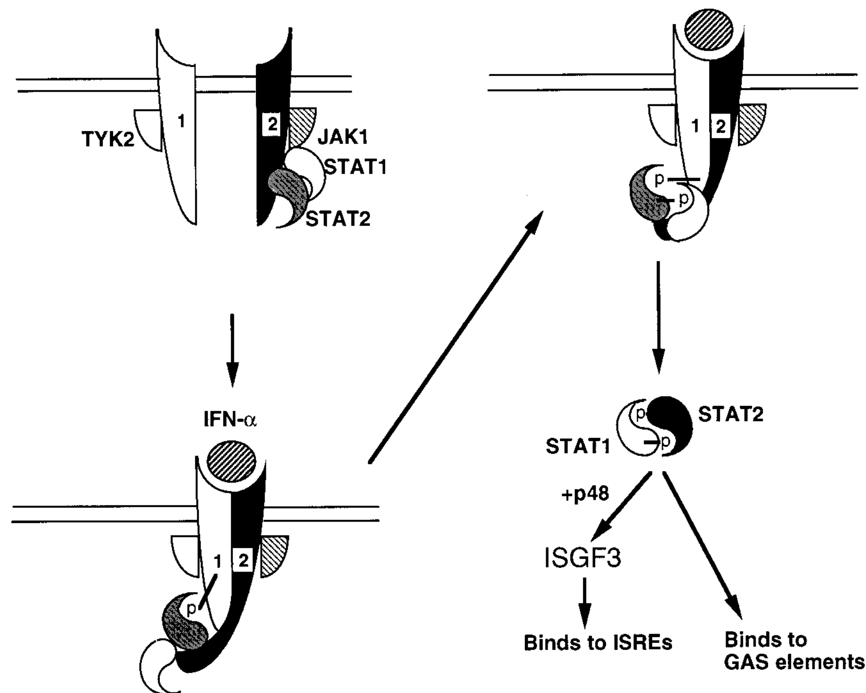


FIG. 10. A model for STAT activation in IFN-α signaling. Signaling components are preassociated with receptor subunits, i.e., TYK2 with IFNAR1 (3) and JAK1 (25), STAT2, and STAT1 with IFNAR2c. STAT2 stabilizes the association of STAT1 with IFNAR2c. IFN-α brings the receptor subunits together, along with the associated JAKs and STATs. Tyr-466 of IFNAR1 is phosphorylated and binds to the SH2 domain of STAT2, which is then phosphorylated on Tyr-690, providing a docking site for the SH2 domain of STAT1. STAT1 is phosphorylated on Tyr-701. The phosphorylated STAT1-STAT2 heterodimer, released by an unknown mechanism, associates with p48 to form ISGF3, which binds to ISREs. The heterodimer can also bind to GAS elements directly.

terminus of STAT1 is required for dephosphorylation of its tyrosine residues. These two functions are mediated by the extreme N terminus of STAT1. The first 50 aa of STAT1 and STAT2 share about 40% identity. Thus, it is possible that the STAT2 N terminus may mediate similar functions, which we are currently investigating. Furthermore, we will determine whether the receptor association domain of STAT2 overlaps with the putative N-terminal domain involved in cooperative STAT binding and dephosphorylation.

Our current model for IFN-α signaling (Fig. 10) has three new features compared to the earlier version (20). First, the receptor subunits and their associated JAKs have been defined better. Two laboratories have shown that TYK2 binds to IFNAR1 (1, 3), and more recently, it has been suggested that JAK1 binds to the cytoplasmic domain of IFNAR2c (25). Second, the docking site for STAT2 has been mapped to p-Tyr-466 of IFNAR1 (this paper and reference 37). Third, we now show that IFNAR2c, STAT2, and STAT1 preassociate and, furthermore, that there is a tight correlation between the binding of STAT2 to IFNAR2c and its activation. Preassociation of STAT1 to the receptor, supported by STAT2, may facilitate STAT1 activation but is not absolutely required. Upon ligand binding, the receptor subunits aggregate, placing all the necessary signaling molecules together (19). Activation of TYK2 and JAK1 leads to phosphorylation of Tyr-466 of IFNAR1, which provides the docking site for the SH2 domain near the C terminus of STAT2. The STAT2-IFNAR1 association probably brings STAT2 to either TYK2 or JAK2 for phosphorylation. (Gupta et al. [12] suggested that, at this point, STAT2 may shift its SH2–p-Tyr interaction from the receptor to the kinases.) Phosphorylated STAT2 then provides the docking site that orients STAT1 for activation. By a yet unknown mechanism, which may require phospholipase A2 (6), STAT1 and STAT2 are then released from the receptor complex as a heterodimer, which can either form ISGF3 with p48 or activate transcription directly upon binding to GAS elements.

ACKNOWLEDGMENTS

The first two authors were equal contributors to this study.

This work was supported by grant CA 62220 from the National Institutes of Health.

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