STAT3 Activation by Type I Interferons Is Dependent on Specific Tyrosines Located in the Cytoplasmic Domain of Interferon Receptor Chain 2c

ACTIVATION OF MULTIPLE STATS PROCEEDS THROUGH THE REDUNDANT USAGE OF TWO TYROSINE RESIDUES*

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Sharlene Velichko‡, T. Charis Wagner‡, James Turkson§, Richard Jove§, and Ed Croze¶

From the ‡Department of Immunology, Berlex Biosciences Inc., Richmond, California 94804 and the §Molecular Oncology and Drug Discovery Programs, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33612

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Human type I interferons (IFNs) play an important role in the regulation of antiviral defense mechanisms, immunomodulatory activities, and growth control. Recent efforts have demonstrated the importance of IFNs in the activation of signal transducers and activators of transcription (STATs). The role of STAT1 and STAT2 in IFN-dependent JAK-STAT signaling is well established; however, the role of STAT3 and its activation by IFNs remains unclear. Understanding the IFN-dependent regulation of STAT3 is of increasing interest because recent studies have demonstrated that STAT3 may play a role in cancer. Studies have revealed that STAT3 is constitutively active in a number of cancer cell lines and that overexpression of an active form of STAT3 transforms normal fibroblasts. Therefore, STAT3 exhibits properties indicative of known oncogenes. In this report, we define the role of the type I IFN receptor in STAT3 activation and identify for the first time tyrosine residues present in the cytoplasmic domain of IFNAR2c that are critical for STAT3 activation. The regulation of STAT3 activation by IFNs was measured in a human lung fibrosarcoma cell line lacking IFNAR2c but stably expressing various IFNAR2c tyrosine mutants. We show here that in addition to IFN-dependent tyrosine phosphorylation of STAT3, activation using a STAT3-dependent electrophoretic mobility shift assay and a STAT3-specific reporter can also be demonstrated. Furthermore, we demonstrate that type I IFN-dependent activation of STAT3 proceeds through a novel mechanism that is dependent on two tyrosines, Tyr337 and Tyr512, present in IFNAR2c and contained within a conserved six-amino acid residue motif, GxGYxM. Surprisingly, both tyrosines were previously shown to be required for type I IFN-dependent STAT1 and STAT2 activation. Our results reveal that type I IFNs activate multiple STATs via the overlapping usage of two tyrosine residues located in the cytoplasmic domain of IFNAR2c.

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† To whom correspondence should be addressed: Dept. of Immunology, Berlex Biosciences, 15049 San Pablo Ave., Richmond, CA 94804. Tel.: 510-669-4043; Fax: 510-669-4246; E-mail: edcroze@berlex.com.
‡ The abbreviations used are: IFN, type I interferon; IFNAR1, human interferon receptor chain 1; IFNAR2c, human type I IFN-α receptor chain 2; EMSA, electrophoretic mobility shift assay; STAT, signal transducer and activator of transcription; JAK, Janus kinase; FACS, fluorescence-activated cell sorter; SH2, Src homology 2; GST, glutathione S-transferase.

Human type I interferons (IFNs)1 play an important role in the regulation of antiviral defense mechanisms, immunomodu-
(12). Other studies have implied that STAT3 binds to the cytoplasmic domain of IFNAR1 in a ligand-dependent manner and serves as an adapter protein in mediating the activation of phosphatidylinositol 3-kinase (18). Studies using chimeric forms of type I and type II IFN receptors expressed in a hamster cell background have demonstrated that the cytoplasmic domain of IFNAR2c may also play a role in STAT3 activation (11); however, the mechanism by which IFNAR2c interacts with and activates STAT3 remains unclear. Because of the emerging role of STAT3 as an important transcription factor (11); however, the mechanism by which IFNAR2c interacts with and activates STAT3 remains unclear. Because of the emerging role of STAT3 as an important transcription factor

**MATERIALS AND METHODS**

Cell Lines and Reagents—The HT1080 cell line was purchased from American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal bovine serum, l-glutamine, and 5% penicillin and streptomycin (Invitrogen) at 37 °C in 5% CO₂. U5A (lacking IFNAR2c) and U6A (lacking STAT2) cells were obtained as described (19). Human IFNβ1b (specific activity 2.5 × 10⁶ units/mg), IFNα2 (specific activity 3.0 × 10⁶ units/mg), and IFNAR2c antiserum were obtained as described previously (6). STAT1, STAT2, STAT3, phosphotyrosine-specific STAT3 (pSTAT3), phosphotyrosine-specific antibodies (pTyr) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Horseradish peroxidase-coupled secondary antibodies were from BIOSOURCE International, Camarillo, CA.

IFNAR2c Tyrosine Mutants—The production and characterization of stable human cell lines expressing IFNAR2c tyrosine mutants (Fig. 1) was described previously (6). Construction and expression of the 337Y512F (Tyr337, Tyr512) and the 337F512F (Tyr269, Tyr306, Tyr316, Tyr337, Tyr341, Tyr348) mutants was performed as follows. The construct expressing the IFNAR2c double tyrosine mutant 337Y512Y was created using pZIFNpR/JLYF (kindly provided by Dr. Oscar Colamonici, Department of Pharmacology, University of Chicago, Chicago, IL) as a template. Single nucleotide substitutions were made via site-directed mutagenesis to create phenylalanine to tyrosine substitutions at amino acid positions 337 and 512. The construct expressing the IFNAR2c mutant 337S512F was created using the Y512F plasmid (6) (kindly provided by Dr. M. Rubinstein, Department of Molecular Genetics and Virology, Weizmann Institute of Science, Rehovot, Israel) as a template for a single nucleotide substitution made via site-directed mutagenesis to create a tyrosine to phenylalanine substitution at amino acid position 337. USA cells (1 × 10⁶ cells/well) were transfected using Superfect transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Multiple stable cell lines for each IFNAR2c mutant were selected in growth media containing G-418 (0.5 mg/ml). After drug selection, cell populations expressing IFNAR2c were further selected using FACS and subcloning as described previously (6). All cell lines were selected to have IFNAR2c receptor levels and ligand binding properties equal to or greater than that observed for HT1080 cells (6). All IFNAR2c tyrosine mutant cell lines are shown in Fig. 1.

Immunoprecipitation and Immunoblotting—STAT1, STAT2, and STAT3 were immunoprecipitated as described previously (5, 6). Briefly, cells (1 × 10⁶ cells) were grown in serum-free media for 1 h and left unstimulated or stimulated for 15 min with either IFN-α or IFN-β (1000 units/10⁶ cells) or IFN-γ (1000 units/10⁶ cells). After IFN treatment, cells were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40 (v/v), 150 mM sodium chloride, 1 mM EDTA, 2.5% glycerol (v/v), 1.0 mM sodium fluoride, 1.0 mM sodium orthovanadate, 1.0 mM phenylmethlysulfonyl fluoride, 0.5 μg/ml leupeptin and 5.0 μg/ml trypsin inhibitor) overnight at 4 °C, incubated with the appropriate antibody, and washed in blocking buffer. For detection of phosphorylated STAT1 and STAT2, the membrane was incubated with a monoclonal phosphotyrosine-specific antibody for 1 h followed by a mouse IgG-specific secondary antibody coupled to horseradish peroxidase-conjugated secondary antibody. After washing, the blot was developed as described previously (6). The immunoblots for STAT1, STAT2, or STAT3, membranes were incubated overnight in 0.01 M sodium citrate, pH 3.0, washed in blocking buffer, and reprobed with the appropriate antibody.

Electrophoretic Mobility Shift Assay (EMSA)—Gel shift assays were performed using 32P-labeled double-stranded oligonucleotides, representing the high affinity sis-inducible element (hSIE, m67 variant, 5′-AGCTTTATTTCTCCGATATCCCTTA-3′) that binds both STAT1 and STAT3 (20). Cells were stimulated with IFN-α (1000 units/10⁶ cells) or IFN-β (1000 units/10⁶ cells) for 15 min, and cell pellets were collected and processed for EMSA as described previously (6). Reaction mixtures were separated by electrophoresis through a 6% polyacrylamide gel and analyzed by autoradiography (21).

RESULTS

Cell lines expressing IFNAR2c tyrosine mutants were previously shown to bind IFN and express IFNAR2c at levels equal to or greater than the parental HT1080 cells by FACs and ligand binding (6). Two new cell lines, one in which Tyr337 and Tyr512 have been substituted to phenylalanine (337F512F) and the other having all tyrosines except Tyr337 and Tyr512 substituted to phenylalanine (337Y512F) were also demonstrated to bind IFN and express IFNAR2c at levels equal to or greater than HT1080 cells (data not shown). IFNAR2c tyrosine mutants used in this study are shown in Fig. 1. Using these cell lines, it was demonstrated that both IFNα and IFNβ induced the phosphorylation of STAT3 in HT1080 and the U5A/2c cell lines (Fig. 2); however, IFNα appeared to be more efficient than IFNβ in activating STAT3 during the 15-min IFN stimulation period. As expected, STAT3 activation was not observed in the U5A or U5A/2F cell lines (Fig. 2). The lack of STAT3 activation in the U5A/2F cells demonstrates that STAT3 activation by type I IFNs is dependent on tyrosine residues present in the intracellular domain of IFNAR2c. In non-serum starved cells, low levels of STAT3 phosphorylation was observed in unstimulated cells suggesting a constitutive level of STAT3 activation (data not shown).

STAT1 activation by IFN was previously shown to be dependent on the presence of STAT2 using a mutant cell line (U6A) deficient in STAT2 (22). It was therefore important to determine whether STAT3 activation by IFNs was also dependent on the presence of STAT2. U6A cells and HT1080 cells were stimulated with either IFNα or IFNβ and activation of STAT1, STAT2, and STAT3 was determined as described under “Materials and Methods.” As expected, U6A cells were unable to induce the phosphorylation of STAT1 after stimulation with IFNα or IFNβ (Fig. 3A). In addition, stripping and reprobing the immunoblot for STAT1 and STAT2 confirmed the lack of STAT2 in U6A cells (Fig. 3A). However, stimulation of
U6A cells with IFN did result in the activation of STAT3 by both IFNα and IFNβ1b, although the level of activation of STAT3 was greater when cells were stimulated with IFNβ1b (Fig. 3B). Therefore activation of STAT3 by type I IFNs does not require the presence of STAT2.

Cell lines stably expressing IFNAR2c in which all but one of the seven tyrosine residues in the cytoplasmic domain have been substituted to phenylalanine were tested for their ability to induce the activation of STAT3 in response to IFN stimulation (Fig. 4). Surprisingly, two tyrosine residues, Tyr337 and Tyr512, which were previously demonstrated to be critical for STAT1 and STAT2 activation (6), were also required for IFN-dependent STAT3 activation (Fig. 4B). To confirm this observation, two additional IFNAR2c tyrosine mutants were analyzed for IFN-dependent STAT3 activation. The first is a mutant in which both Tyr337 and Tyr512 were substituted with phenylalanine (337F512F). The second mutant (337Y512Y) has all cytoplasmic tyrosines substituted with phenylalanine except Tyr337 and Tyr512. IFN stimulation of these cell lines revealed that STAT3 activation was observed only in 337Y512Y and not in 337F512F, ruling out the involvement of other tyrosine residues present in IFNAR2c (Tyr269, Tyr306, Tyr316, Tyr318, and Tyr411) in STAT3 activation (Fig. 4C). This observation is in agreement with previous results demonstrating the dependence on Tyr337 and Tyr512 in the activation of STAT1 and STAT2 by IFN (6).

IFN-dependent activation of STAT3 leads to the formation of homo- and heterodimeric transcription factors such as STAT3:STAT3 and STAT3:STAT1, which induce gene transcription upon binding to specific DNA elements in the promoter region of a number of genes (23, 24). One such high-affinity DNA binding site for STAT3 is the m67 element (13). We next sought to determine whether IFN-dependent tyrosine phosphorylation of STAT3 leads to the formation of STAT3 transcription complexes that bind specific high-affinity STAT3 DNA binding elements. To investigate this possibility, EMSAs were per-
IFN-dependent STAT3 Activation by Type I IFN Receptor

IFN was observed in the parental HT1080 and U5AR2c cell lines. As expected, formation of STAT3 transcription complexes was not observed in U5A or U5A7F cells upon IFN stimulation (Fig. 5). However, a strong induction of STAT3 transcription complexes was observed in the 337Y512F cell line, whereas none was detected in the 337F512F cell line, demonstrating the importance of Tyr337 and Tyr512 in STAT3 transcription complex formation. Low levels of constitutive STAT3 activation were occasionally observed in unstimulated cells. Identification of STAT3:STAT3 and STAT3:STAT1 protein-DNA complexes were confirmed by STAT3- or STAT1-specific supershift assay (data not shown).

The dependence on Tyr337 and Tyr512 in STAT3 activation by IFN was further demonstrated by determining the IFN-dependent activation of a STAT3-specific reporter in various IFNAR2c tyrosine mutant cell lines. To demonstrate the induction of this reporter by IFN, cell lines expressing IFNAR2c tyrosine mutants were transiently transfected with an m67-luciferase reporter; after IFN stimulation the extent of induction of the reporter was determined (Fig. 6). As expected, U5A and U5A7F cells were unable to induce activation of the m67-luciferase reporter after IFN stimulation, further supporting the observation that STAT3 activation requires IFNAR2c and proceeds through a mechanism involving cytoplasmic tyrosine residues present in this receptor chain. However, HT1080, U5AR2c, 337Y, and 512Y cells all strongly induced expression of the STAT3-dependent reporter in response to IFN stimulation (Fig. 6). Finally, the importance of Tyr337 or Tyr512 in STAT3-dependent induction of the m67-luciferase reporter was confirmed by the observation that efficient reporter activation was observed only in the tyrosine mutant 337Y512F and not in the 337F512F cell line (Fig. 6).

**DISCUSSION**

The STAT family of proteins consist of seven different members, many of which can be activated directly by cytokines and growth factors. STAT proteins are involved in a number of important biological responses including, but not limited to, innate and adaptive immune responses, cell growth and regulation, apoptosis, and antiviral responses (1, 16, 26). One common feature within the protein structure of all STATs is the presence of a Src homology 2 (SH2) domain. SH2 domains bind specifically to phosphotyrosines and can thereby mediate the recruitment of STATs to the cytoplasmic domain of cell surface receptors. In addition, phosphorylation of STATs themselves leads to their homo- or heterodimerization, subsequent nuclear localization, and activation of transcription. The activation of STATs is tightly controlled by tyrosine phosphorylation, and the control of STAT activation appears to be of universal importance in that similar STAT activation mechanisms occur in organisms ranging from slime molds to humans (23). Recently, STAT1, STAT3, and STAT5 have been implicated in a number of cancer types (1, 16, 24). STAT3 itself has been

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**Fig. 3.** STAT3 activation by IFN occurs in the STAT2-deficient U6A cell line and is not dependent on the presence of STAT2. HT1080 and U6A cell lines were serum-starved for 1 h and then left untreated (0) or stimulated for 15 min with IFNα (α) or IFNβ (β) (1000 units/10^6 cells). After stimulation, cells were solubilized in lysis buffer, and STAT1, STAT2, or STAT3 were immunoprecipitated. Immunoblotting and immunoblot reprobing was as described for Fig. 1. The locations of STAT1, STAT2, and STAT3 are indicated. A, STAT1 and STAT2 activation in HT1080 and U6A cells. B, immunoprecipitating STAT1 and STAT2 polyclonal antibody; BLOT: pTyr, antibody specifically recognizing phosphorytrosine residues; BLOT: STAT1 & 2, immunoblotting monoclonal antibodies recognizing either STAT1 or STAT2. C, STAT3 activation in HT1080 and U6A cells. IP, immunoprecipitating STAT3 polyclonal antibody; BLOT: pSTAT3, antibody specifically recognizing only the tyrosine-phosphorylated form of STAT3; BLOT: STAT3, immunoblotting monoclonal antibody.

**Fig. 4.** IFN-dependent STAT3 activation proceeds through the redundant usage of Tyr337 and Tyr512. Cells were serum-starved for 1 h and left untreated (0) or stimulated with IFNα (α) or IFNβ (β) (1000 units/10^6 cells) for 15 min, solubilized in lysis buffer, and STAT3-immunoprecipitated. Following immunoprecipitation, tyrosine phosphorylation of STAT3 was determined by phosphotyrosine immunoblotting using a STAT3 antibody specifically recognizing only STAT3 phosphorylated at Tyr512 (pSTAT3). The position of STAT3 is indicated. After detection of phosphorylated STAT3, the membrane was stripped and reprobed with a STAT3-specific monoclonal antibody. IP, immunoprecipitating STAT3 polyclonal antibody; BLOT: pSTAT3, monoclonal antibody specifically recognizing phosphorylated Tyr512 in STAT3. BLOT: STAT3, immunoblotting monoclonal antibody. Cell lines were as described for Fig. 1.

**Fig. 5.** STAT3 transcription complex formation requires IFNAR2c Tyr337 or Tyr512. Cells were left untreated (0) or stimulated with IFNα (α) or IFNβ (β) (1000 units/10^6 cells) for 15 min as indicated. Cell pellets were then harvested and processed for EMSA. EMSAs using hSIE m67 variant probes were performed as described under "Material and Methods." The locations of STAT3, STAT1, and STAT1:1 transcription complexes are indicated by the arrows. Cell lines are as described under "Materials and Methods" or as for Fig 1.

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**Table 1.** Summary of STAT3 Dimerization Studies

| Dimerization | U6A | HT1080 |
|--------------|-----|--------|
| STAT3:STAT3  | 0   | α, β   |
| STAT3:STAT1  | 0   | α, β   |
| STAT3:STAT2  | 0   | α, β   |

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**Table 2.** Summary of STAT3 Supershift Assays

| Supershift | U6A | HT1080 |
|------------|-----|--------|
| STAT3      | 0   | α, β   |
| STAT1      | 0   | α, β   |
| STAT2      | 0   | α, β   |
various IFNAR2c tyrosine mutants in the absence (●) or presence (+) of IFN-β (1000 units/ml) as described under “Materials and Methods.” Cell lines were transiently co-transfected with a plasmid containing the m67-luciferase reporter or the Renilla luciferase reporter (pRLTK). Relative luciferase units expressed on the y axis are mean values ± the standard deviation performed in quadruplicate and normalized to the receptor (32). This observation parallels those made in the present study. The recognition of identical tyrosines residues by different STATs strongly suggests that such tyrosine residues are uniquely located within the cytoplasmic domain of these receptors and are likely unhindered substrates for receptor-associated tyrosine kinases or docking sites for STATs, adapter proteins, or certain phosphatases.

It has been shown previously that IFN-dependent activation of STAT1 was inhibited in a mutant human cell line (U6A) lacking STAT2 (22). Such an observation demonstrates the requirement for STAT2 for STAT1 activation and suggests a mechanistic association between STAT1 and STAT2. In the present study we show that STAT3 activation occurs unabated in U6A cells, demonstrating that STAT3 activation is not dependent on the presence of STAT2. Therefore, although Tyr337 and Tyr512 are required for activation of all three STATs the exact mechanism of activation for STAT1 and STAT2 may be somewhat different from that observed for STAT3. Distinct mechanisms of activation of STAT1, STAT2, and STAT3 by IFNs is supported by recent studies in which it was shown that piceatannol, a tyrosine kinase inhibitor, inhibited the IFN-dependent tyrosine phosphorylation of STAT3 and STAT5 but not of STAT1 and STAT2 in a Burkitt’s lymphoma human B cell line (33).

The absolute requirement of either Tyr337 and Tyr512 for STAT1, STAT2, and STAT3 activation suggests a specific interaction of these STATs with Tyr337 and Tyr512. Both Tyr337 and Tyr512 are separated by a stretch of 175 amino acid residues, placing these amino acid residues at the distal end of the IFNAR2c receptor chain. Examination of the amino acid residues present in this region failed to identify a STAT3 tyrosine-based activation motif (YxxQ) similar to one previously found associated with IFNAR1 and gp130. Surprisingly, a previously unidentified conserved amino acid motif, GxGxxM, was found associated with both Tyr337 and Tyr512 and not with any other tyrosine residue present in the cytoplasmic domain of IFNAR2c or IFNAR1 (Fig. 7). A data base search using this protein motif revealed no significant sequence alignments with other known motifs.

Although STATs are large proteins relative to the cytoplasmic domain of either IFN receptor chain, it is possible that more than one STAT can interact with IFNAR2c simultaneously or in rapid succession allowing for efficient STAT1, STAT2, and STAT3 activation. The simplest model for IFN-dependent STAT activation would first involve the binding of Tyr337 and Tyr512. However, the role of specific tyrosines present in the cytoplasmic domain of IFNAR2c in the activation of STAT3 remained to be established.

To address this question we made use of IFNAR2c tyrosine mutants stably expressed in a human cell background to identity tyrosine residues important for STAT3 activation. Analysis of a number of IFNAR2c tyrosine mutants demonstrated that two tyrosines, Tyr337 and Tyr512, previously shown to be important for STAT1 and STAT2 activation were also required for STAT3 activation. The role of these two tyrosine residues in STAT3 activation was demonstrated by different methods including the measurement of STAT3 phosphorylation, formation of STAT3 transcription complexes, and the IFN-dependent induction of a STAT3 reporter. Collectively these results all demonstrate the requirement for Tyr337 and Tyr512 in STAT3 activation. Multiple STAT activation via the overlapping usage of specific tyrosine residues within the same receptor chain has been demonstrated previously for other receptors. An example of this is the human growth hormone receptor, in which activation of STAT1, STAT3, and STAT5 requires the presence of either Tyr333 or Tyr338 are located in the cytoplasmic domain of the receptor (32). This observation parallels those made in the present study. The recognition of identical tyrosines residues by different STATs strongly suggests that such tyrosine residues are uniquely located within the cytoplasmic domain of these receptors and are likely unhindered substrates for receptor-associated tyrosine kinases or docking sites for STATs, adapter proteins, or certain phosphatases.

suggested to have oncogenic properties and is constitutively activated in a number of tumor cell lines (13). Although STAT3 and STAT1 are highly homologous, they appear to have divergent functions. An example of this is the observation that in knock-out mice STAT3 but not STAT1 is embryonic lethal (27). In addition, it has been shown that STAT3 and not STAT1 is required for their activation, serine phosphorylation also appears to be required for maximal transcriptional activity (29).

IFN-dependent STAT3 activation has been documented in a number of studies, but the mechanism of activation by IFNs remains unclear. Previous studies demonstrated that STAT3 interacts with IFNAR1 and mediates the subsequent activation of the phosphatidylinositol 3-kinase signaling pathway (18). Further evidence supporting the interaction of IFNAR1 and STAT3 was provided by the discovery of a specific STAT3 motif, YxxQ, present in the cytoplasmic domain of IFNAR1. This motif was shown to be responsible for mediating the interaction between STAT3 and gp130 (30, 31). Recent studies have extended these observations to demonstrate that IFN-dependent phosphorylation of IFNAR1 and STAT3 requires the Janus tyrosine kinase TYK2 (12). However, other studies have called into question the exact role of IFNAR1 in the activation of both STAT2 and STAT3 by demonstrating that switching the entire cytoplasmic domain of IFNAR1 with that of another receptor (11) or substituting all cytoplasmic tyrosines to phenylalanines (9) had no effect on STAT activation.

Early events in IFN signaling require ligand-dependent receptor assembly, receptor phosphorylation, and STAT activation. Other studies have extended these observations to demonstrate the requirement of specific tyrosine residues present in the cytoplasmic domain of IFNAR2c in both STAT1 and STAT2 activation. In these studies it was suggested that elimination of both Tyr337 and Tyr512 could result in a receptor unable to activate STAT1 or STAT2 in response to IFN stimulation (6). These results showed that IFN-dependent activation of STAT1 and STAT2 proceeds through the redundant usage of Tyr337 and Tyr512. However, the role of specific tyrosines present in the cytoplasmic domain of IFNAR2c in the activation of STAT3 remained to be established.
STATs to phosphorylated Tyr$^{337}$ or Tyr$^{512}$ through an interaction with the STAT-SH2 domain. Once bound to the receptor chain, the STATs themselves become substrates for the receptor-associated Janus tyrosine kinases and become phosphorylated on specific tyrosines. Phosphorylated STATs then associate with each other as homo- or heterodimers, dissociate from the receptor, and translocate into the cell nucleus initiating gene expression. However, recent studies using GST-STAT or GST-IFNAR2c constructs to co-precipitate STAT1 and STAT2 suggest that STAT1 and STAT2 preassociate with IFNAR2c in a ligand-independent manner (34, 35). These studies suggest that STAT1 and STAT2 initially interact with IFNAR2c independently of SH2 domain interactions via protein-protein interactions yet to be completely defined. Therefore, the role of specific IFNAR2c-associated phosphotyrosines in JAK-STAT signaling could be independent of any initial receptor docking event.

It is clear that tyrosines present in the cytoplasmic domain of IFNAR2c are required for IFN signaling and STAT activation (6). Accordingly, the interaction of STATs with phosphorylated tyrosines on IFNAR2c could occur after STAT docking to the IFN receptor. Regardless of the role of Tyr$^{337}$ and Tyr$^{512}$ in the docking of STATs to the IFN receptor, regulation of JAK-STAT signaling is likely to be achieved in part through the interaction of the STAT-SH2 domain with these tyrosines. We propose that such an interaction leads to a conformational change within the STAT protein itself in which specific tyrosines, most likely STAT1, Tyr$^{701}$, STAT2, Tyr$^{689}$, and STAT3, Tyr$^{705}$ (23) become high affinity substrates for the receptor-associated Janus tyrosine kinases JAK1 or TYK2. The tyrosine phosphorylation of receptor-associated STATs would then allow for their homo- or heterodimerization, dissociation from the receptor, and induction of IFN-responsive genes. It is also likely that these tyrosines play an additional role in mediating IFN signaling by interacting with adapter molecules, phosphatases, or other kinases. A model outlining a proposed mechanism of STAT activation by IFN is shown in Fig. 8. The docking of STAT proteins to specific IFNAR2c tyrosine mutants is currently under investigation. Results from these studies should help to further define the role of IFNAR2c tyrosine residues in mediating the direct docking and subsequent activation of STATs by IFN.

A number of unanswered questions still remain concerning the mechanism of activation of STATs by IFNs. Specific adapter proteins have been implicated in regulating the association of STATs with various cytokine receptors or associated kinases (18, 36–38). Therefore, it is possible that although Tyr$^{337}$ and Tyr$^{512}$ are required for STAT activation the differences observed between the activation of STAT1, STAT2, and STAT3 in U6A cells may be explained by a required interaction of a particular STAT protein with an adapter molecule. Furthermore, an interaction of the IFN receptor with gp130, which is known to mediate STAT3 activation, has also been suggested (25). If such an interaction does indeed occur, it would provide yet another possible mechanism by which STAT3 could initially associate with the IFN receptor prior to activation.

Finally, our results clearly identify for the first time the importance of Tyr$^{337}$ and Tyr$^{512}$ in the activation of STAT3 by IFN. STAT3 activation and receptor function is achieved when either Tyr$^{337}$ or Tyr$^{512}$ is present, demonstrating that a single tyrosine is able to sustain normal STAT3 activation and IFN signaling. Furthermore, this is the first demonstration of the overlapping usage of cytoplasmic tyrosines by the IFN receptor in the activation of multiple STATs, such as STAT1, STAT2, and STAT3. In addition, this work further supports the observation that IFN-dependent signaling through the JAK-STAT pathway proceeds through the redundant usage of two tyrosines residues in the cytoplasmic domain of IFNAR2c.
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