Contribution of STAT SH2 Groups to Specific Interferon Signaling by the Jak-STAT Pathway

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In response to specific ligands, various STAT proteins (signal transducers and activators of transcription) are phosphorylated on tyrosine by Jak protein kinases and translocated to the nucleus to direct gene transcription. Selection of a STAT at the interferon γ receptor as well as specific STAT dimer formation depended on the presence of particular SH2 groups (phosphotyrosine-binding domains), whereas the amino acid sequence surrounding the phosphorylated tyrosine on the STAT could vary. Thus, SH2 groups in STAT proteins may play crucial roles in specificity at the receptor kinase complex and in subsequent dimerization, whereas the kinases are relatively nonspecific.

By binding to their cell surface receptors, many different polypeptides specifically activate as DNA-binding proteins one or more members of a family of latent transcription factors termed STATs (*1*). STAT activation results initially from the tyrosine phosphorylation and consequent activation of Jak protein kinases (*2–4*) that are already associated with cell surface receptors (*4–6*), after which the STAT proteins are phosphorylated on tyrosine, homo- or heterodimerize (*7, 8*), and translocate to the cell nucleus to direct transcriptional responses. Interferon α (IFN-α) induces the activation of Jak1 and Tyk2 protein kinases (*2–4, 9*) followed by the phosphorylation of Stat1 and Stat2 (*10*), whereas IFN-γ induces activation of Jak1 and Jak2 (*2–4, 9*) followed by the phosphorylation of only Stat1 (*11*). It remains unknown how the specificity of the signal is maintained during its transduction through the receptors and kinases to the STATs, although Tyr^{440} on one of the IFN-γ receptor chains is crucial for IFN-γ activation. In addition, a phosphopeptide encompassing Tyr^{440} can specifically bind Stat1 (*12, 13*).

We swapped between Stat1 and Stat2 either the SH2 (phosphotyrosine binding) domains or the sites for phosphorylation of tyrosine. The SH2 domain of Stat1 as part of either the Stat1 or Stat2 molecule directed phosphorylation at the IFN-γ receptor, whereas the SH2 domain of Stat2 did not. The target tyrosine residue (Tyr^{701} in Stat1 and Tyr^{690} in Stat2) and surrounding sequence were also swapped, and either the Stat1 or Stat2 sequence could be phosphorylated at either receptor on condition that a Stat1 SH2 domain was provided. Further, two molecules with a Stat1:2 or Stat1:1 SH2 combination can dimerize, but the presence of the Stat2 SH2 group in both potentially interacting molecules blocks interaction. We conclude that the STAT-SH2 group is crucial in providing specificity at the IFN-γ receptor and also directs dimerization of activated STATs and that the Jak kinases activated at the IFN-α or IFN-γ receptors are not highly specific.

To assay for the functions of the Stat1 and Stat2 SH2 domains, we prepared recombinant DNA constructs that encoded the hybrid molecules (Fig. 1). We used the abbreviations Stat1-(SH2)2 or Stat2-(SH2)1 to indicate swapped SH2 domains inserted in the recipient molecule with borders chosen to match the canonical SH2 domains. In addition to the SH2 domains, the tyrosine phosphorylation sites (abbreviated Y) were swapped to determine whether the kinases resident at the two receptors could distinguish phosphorylation sites of different STATs. The recombinant constructs were transfected along with a selectable antibiotic resistance marker into appropriate cell lines—U3A (lacking Stat1) (*14*) or U6A (lacking Stat2) (*15*)—and permanent cloned cell lines were selected.

In the first set of experiments (Fig. 2A), Stat1 and Stat1-(SH2)2 tagged with the FLAG epitope [an eight–amino acid epitope recognized by monoclonal antibody M2 (*16*)] were expressed independently in U3A cells. As determined by the phosphotyrosine content of antibody to FLAG (M2)–precipitated protein, wild-type Stat1 was activated by either IFN-α or IFN-γ (Fig. 2A). In contrast, the Stat1-(SH2)2 protein was activated only by IFN-α (Fig. 2A), suggesting that the SH2 group of Stat2 was not able to bring this chimeric molecule into proximity of the IFN-γ receptor. Another conclusion from this experiment bears on STAT dimerization after IFN treatment. Precipitation of IFN-α–treated cell extracts resulted in the coprecipitation of the FLAG-tagged wild-type Stat1 with the endogenous Stat2 that had been activated (phosphorylated). In contrast, the FLAG-tagged IFN-α–activated Stat1-(SH2)2 did not interact with the endogenous Stat2. Immunoprecipitation of the same extracts with antiserum to Stat2 verified that the same amount of activated Stat2 was present in both cell types (*17*). Thus, the Stat2 SH2 group of the Stat1-(SH2)2 construct does not interact with the endogenous Stat2 even though both molecules are phosphorylated. This result is consistent with the absence of Stat2 homodimers in unsupplemented U3A cells (*18*) treated with IFN-α where Stat2 is phosphorylated—or, for that matter, in any cell examined thus far (*17*).

If Stat2 is not activated by IFN-γ because its SH2 group cannot bind to the phosphorylated IFN-γ receptor, but activation of the native Stat1 depends on the

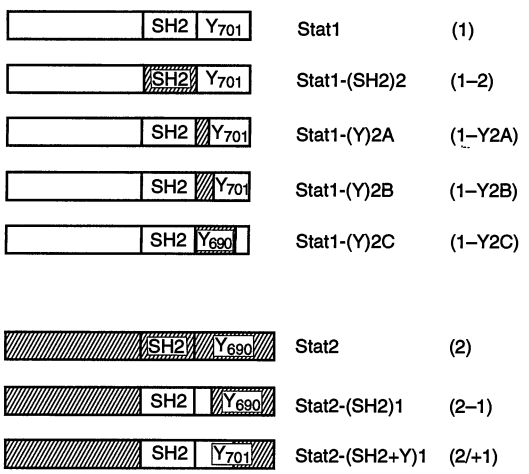

Fig. 1. Stat1, Stat2, and Stat1-Stat2 chimeras. The constructs were made with chimeric oligonucleotides and through use of the polymerase chain reaction with Vent polymerase (New England Biolabs). All clones were sequenced through the chimeric regions and the start and stop codons. The constructs were cloned into either pcDNA3 (Invitrogen) or pBactNeo (*24*). Stat1 and Stat1-(SH2)2 were tagged with the FLAG epitope at the COOH-terminus (*16*). The amino acid (aa) boundaries of the chimeric proteins are as follows: Stat1-(SH2)2 has aa 1 to 572 of Stat1, 572 to 668 of Stat2, and 672 to 750 of Stat1; Stat1-(Y)2A has aa 1 to 671 of Stat1, 669 to 682 of Stat2, and 686 to 750 of Stat1; Stat1-(Y)2B has aa 1 of 671 of Stat1, 669 to 682 of Stat2, and 692 to 750 of Stat1; Stat1-(Y)2C has aa 1 to 671 of Stat1, 669 to 699 of Stat2, and 711 to 750 of Stat1; Stat2-(SH2)1 has aa 1 to 571 of Stat2, 573 to 700 of Stat1, and 690 to 851 of Stat2; and Stat2-(SH2+Y)1 has aa 1 to 571 of Stat2, 573 to 710 of Stat1, and 700 to 851 of Stat2. Abbreviation used in Figs. 2 and 3 are shown to the right. Y, tyrosine phosphorylation site.

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presence of the Stat1 SH2 group, then a Stat2 chimera with the SH2 group of Stat1 should be activated by IFN-γ. A Stat2 protein containing the Stat1 SH2 domain (amino acids 573 to 671) was not activated by either IFN-α or IFN-γ (*17*). However, this molecule also could not be activated under conditions that nonspecifically activate STAT proteins. Overexpression of STATs together with Jaks in COS cells leads to activation of Stat1 and Stat2 in the absence of ligand (*19*), but did not result in phosphorylation of this chimeric protein. We therefore constructed plasmids to encode two chimeric proteins with Stat1 SH2 domains that contain extra amino acids toward the COOH-terminus from the SH2 domain (Fig. 1). Stat2-(SH2)1 includes amino acids of Stat1 (residues 573 to 700) but still has the tyrosine phosphorylation site and COOH-terminal sequences of Stat2. Stat2-(SH2+Y)1 contains a 137–amino acid region (amino acids 573 to 710) from Stat1 that includes the SH2 domain and the tyrosine phosphorylation site of Stat1 in place of the SH2 and tyrosine of Stat2. Both constructs were activated by IFN-α and IFN-γ (Fig. 2B) and both proteins were immunoprecipitated along with endogenous Stat1 by an antiserum to Stat1.

U3A cells that lack Stat1 of course show no response to IFN-γ, but Stat2 is still phosphorylated on the correct tyrosine in response to IFN-α (*18*). In contrast, in U6A cells that lack Stat2, Stat1 is not phosphorylated in response to IFN-α but functions normally in response to IFN-γ (*15*). Thus, Stat1 activation in U6A cells at the IFN-α receptor-kinase complex requires the presence of Stat2. When the Stat1-(SH2)2 construct was expressed in U6A cells, it was not phosphorylated in response to IFN-α, nor was phosphorylation of the endogenous Stat1 restored (*20*). Thus, the SH2 group of Stat2 incorporated into Stat1 cannot substitute for all of the functions required of Stat2 at the IFN-α receptor. However, the Stat2 SH2 group is essential both for Stat2 and for Stat1 phosphorylation in response to IFN-α. For example, a crucial arginine in the phosphotyrosine-binding cavity of the Stat2 SH2 group was mutated, and the mutant was introduced into U6A cells; the activation of both the mutant Stat2 and the endogenous Stat1 was abolished (*21*). In addition, one chain in the IFN-α (*22*) and the IFN-γ receptor (*12*) acquires a phosphate on tyrosine in a ligand-dependent manner. Thus, we believe that the Stat2 SH2 group is necessary for Stat2 and Stat1 phosphorylation at the IFN-α receptor, but it is not by itself sufficient.

The IFN-α pathway requires Tyk2 and Jak1, whereas the IFN-γ pathway requires Jak2 and Jak1 (*2–4*). Such results could mean that Stat2 phosphorylation depends specifically on the presence of Tyk2 in a receptor-kinase complex, because Stat2 is activated only after IFN-α and not IFN-γ treatment. However, the activation of Stat2-(SH2)1 at the IFN-γ receptor (Fig. 2B) suggests that Tyk2 is not specifically required for phosphorylation of the Stat2 tyrosine site.

To further test the importance of amino acid sequences surrounding the tyrosine substrate sites for the phosphorylation by Jak1 or Jak2 at the IFN-γ receptor, we made three Stat1 constructs—Stat1-(Y)2A, Stat1-(Y)2B, and Stat1-(Y)2C (Fig. 1)—that contained Stat2 sequences upstream and downstream of the tyrosine phosphorylation site. The constructs were transfected into U3A cells, and clonal lines were developed and assayed. Treatment with either IFN-α or IFN-γ activated these recombinant proteins approximately equally, indicating that the Stat2 tyrosine surrounded by the Stat2 amino acid sequence could be phosphorylated by Jak1 or Jak2 at the IFN-γ receptor and by Tyk2 or Jak1 at the IFN-α receptor (Fig. 3).

Our results show that swapping the SH2 domain from Stat1 into Stat2 allowed activation of the recombinant protein at the IFN-γ receptor, and insertion of the SH2 group of Stat2 into Stat1 prevented activation of the recombinant protein at the IFN-γ receptor. Thus, the specificity for activation at the IFN-γ receptor resides in the Stat1 SH2 group, whereas the IFN-α receptor will accept molecules with either SH2 group. In contrast, swapping the substrate tyrosine and surrounding amino acids between Stat1 and Stat2 did not change the specificity of activation by IFN-α and IFN-γ. Thus, the Jak protein kinases are not the source of specificity in the Jak-STAT pathway. Further, dimerization of Stat1 and

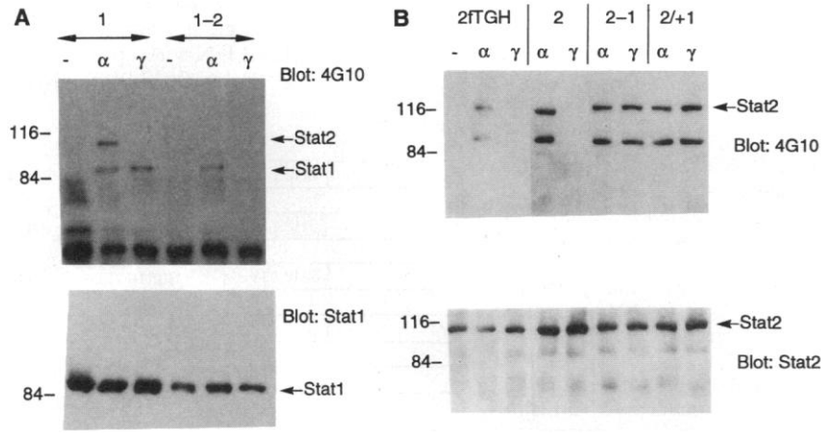

Fig. 2. Specification of STAT-receptor interactions by SH2 domains. (**A**) U3A cells expressing the FLAG-tagged Stat1 (1) or the chimeric Stat1-(SH2)2 with the SH2 domain of Stat2 (1–2) were left untreated (−) or treated for 15 min with either IFN-α (α; 500 IU/ml) or IFN-γ (γ; 5 ng/ml). (Upper panel) Extracts were precipitated with a monoclonal antibody to FLAG (M2) bound to a gel (Kodak), subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (7% gel), and probed with a monoclonal antibody (4G10) to phosphotyrosine (UBI). (Lower panel) After stripping, the gels were reprobed with antiserum to Stat1 (*25*). (**B**) The parental cell line 2fTGH or U6A cells expressing wild-type Stat2 (2) or the Stat2-(SH2)1 or Stat2-(SH2 + Y)1 constructs were left untreated (−) or treated for 15 min with either IFN-α (α; 500 IU/ml) or IFN-γ (γ; 5 ng/ml). (Upper panel) Extracts were precipitated with an antiserum to Stat2 (*26*). The precipitates were probed with a monoclonal antibody (4G10) to phosphotyrosine (UBI). (Lower panel) After stripping, the blots were probed with antiserum to Stat2 (*27*).

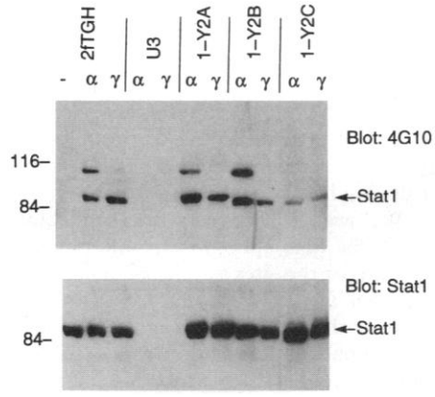

Fig. 3. Phosphorylation of Tyr^{690} of Stat2 by kinases associated with the IFN-γ receptor. 2fTGH cells, U3A cells, or U3A cells transfected with chimeric Stat1 proteins containing amino acids of Stat2 starting after the SH2 domain and extending toward (1–Y2A and 1–Y2B) or beyond (1–Y2C) Tyr^{690} of Stat2 were treated for 15 min with either IFN-α (α; 500 IU/ml) or IFN-γ (γ; 5 ng/ml). Proteins from extracts were precipitated with antiserum to Stat1 (*25*). The precipitates were subjected to SDS-PAGE (7% gel) and probed with antibody to phosphotyrosine (4G10) and, after stripping of the blots, with antiserum to Stat1. Transfections, selection, maintenance, lysis, and sample handling were as described in Fig. 2.

Stat2 recombinant molecules required either two Stat1-SH2 groups or one Stat1- and one Stat2-SH2 group; two Stat2-SH2 groups did not permit interaction, even though the two potentially interacting proteins were phosphorylated.

The following model for the operation of the Jak-STAT pathway is supported by these and other data (*1*). Ligand-driven receptor-kinase assembly occurs between receptor chains and associated Jak protein kinases. Phosphorylation (intermolecular) of the Jaks ensues, and these active enzymes phosphorylate one or more of the receptor chains. The substrate STAT molecule is directed by its SH2 group to a tyrosine phosphate on a specific receptor-kinase complex. Binding of the SH2 group brings the closely neighboring tyrosine on the STAT protein into proximity of a kinase. If the SH2 domains and tyrosine sites of the phosphorylated STATs are mutually compatible, the proteins form homo- or heterodimers. After translocation into the nucleus, specific binding to DNA, alone or in conjunction with other proteins, directs specific transcriptional responses. This set of specific protein-protein and protein-DNA interactions allows a distinct response to a particular extracellular signaling protein.

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27. U3A cells (5×10^5) (*14*) or U6A cells (*15*) were transfected with DNA (20 μg) by the calcium phosphate procedure (*23*). Colonies were selected in Dulbecco's modified Eagle's medium supplemented with bovine calf serum containing G418 (500 μg/ml; Gibco, BRL) 48 hours after transfection. Cell lines were maintained in G418. Cells were lysed for 30 min on ice in 50 mM tris (pH 8.0), 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4, leupeptin (1 μg/ml), aprotinin (2 μg/ml), and pepstatin (1 μg/ml). Precipitates were washed twice in lysis buffer and once in phosphate-buffered saline, then boiled for 2 min in sample loading buffer [50 mM tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1% β-mercaptoethanol]. Precipitates were subjected to SDS-PAGE (7% gel), transferred to nitrocellulose membranes, and probed with the antisera indicated above.

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Choice of STATs and Other Substrates Specified by Modular Tyrosine-Based Motifs in Cytokine Receptors

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Many members of the cytokine receptor superfamily initiate intracellular signaling by activating members of the Jak family of tyrosine kinases. Activation of the same Jaks by multiple cytokines raises the question of how these cytokines activate distinct intracellular signaling pathways. Selection of particular substrates—the transcriptional activator Stat3 and protein tyrosine phosphatase PTP1D—that characterize responses to the ciliary neurotrophic factor–interleukin-6 cytokine family depended not on which Jak was activated, but was instead determined by specific tyrosine-based motifs in the receptor components—gp130 and LIFR—shared by these cytokines. Further, these tyrosine-based motifs were modular, because addition of a Stat3-specifying motif to another cytokine receptor, that for erythropoietin, caused it to activate Stat3 in a ligand-dependent fashion.

The cytokine receptor superfamily comprises structurally related receptors characterized by conserved motifs in their ectodomains (*1*). Many different cytokines—with biological actions as distinct as those of erythropoietin (EPO), ciliary neurotrophic factor (CNTF), and the interferons (IFNs)—activate members of this receptor superfamily (*2, 3*). The cytoplasmic domains of cytokine receptors lack obvious catalytic function. However, binding of ligand to the cytokine receptors initiates intracellular signaling by activating members of a family of receptor-associated tyrosine kinases, referred to as the Janus kinases (Jaks) (*2, 4*). These kinases are constitutively associated with the membrane-proximal portions of cytokine receptor cytoplasmic domains and become activated upon ligand-induced receptor homo- or heterodimerization (*2, 5, 6–10*), a process reminiscent of epidermal growth factor (EGF)– and platelet-derived growth factor (PDGF)–mediated dimerization and subsequent activation of receptor tyrosine kinases (*11*). EPO causes homodimerization of its receptor, which leads to specific activation of the attached kinase, Jak2 (*7*). The CNTF family of cytokines, including interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and oncostatin M (OSM), use more complicated receptor systems that contain two different Jak-associated receptor components, gp130 and LIFR (*2*). The CNTF and IL-6 receptor complexes also include α receptor components that confer cytokine specificity; these α components do not bind to Jaks and their cytoplasmic domains are apparently not required for signaling (*2*). Ligand-mediated activation results from either homodimerization of gp130 for IL-6 or heterodimerization of gp130 with LIFR for CNTF, LIF, and OSM (*8, 9*). Both gp130 and LIFR can associate with and activate at least three members of the Jak family—Jak1, Jak2, and Tyk2—but activate distinct combinations of these Jaks in different cells (*10*). Although the receptor systems for the IFNs are not fully understood, signaling by IFN-α requires Jak1 and Tyk2, whereas signaling by IFN-γ specifically requires Jak1 and Jak2 (*12–14*).

It is not clear how different cytokines, often activating the same Jaks, can uniquely specify activation of distinct intracellular

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