The EMBO Journal vol.15 no.5 pp.1075–1084, 1996

The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-α signals

Sanjay Gupta[1], Hai Yan[2], Lee Hwa Wong[3], Steve Ralph[3], John Krolewski[2] and Christian Schindler[1,4,5]

[1]Departments of Medicine and Physiology and Cellular Biophysics, College of Physicians and Surgeons, [2]Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA and [3]Department of Biochemistry and Molecular Biology, Monash University, Clayton VIC3 168, Australia

[4]Present address: Columbia University, Division of Molecular Medicine, PH 8 East, 630 West 168th Street, New York, USA

[5]Corresponding author

Analysis of the ability of IFN-α to rapidly stimulate genes has led to the identification of a new family of signal transducing factors (STFs). The STF activated by IFN-α consists of two members of the STAT (Signal Transducers and Activators of Transcription) family of signaling proteins, Stat1 and Stat2. Sequence comparison of these STATs has determined that they share several conserved domains, the most notable of which is an SH2 domain. Recently, studies have determined that these SH2 domains can mediate a specific association between a STAT and the cytoplasmic domain of the appropriate receptor. Once associated with the receptor, the STATs become activated by an associated tyrosine kinase, whereupon they dissociate from the receptor and dimerize to form active STFs. The SH2 domain has also been implicated in the formation of STAT dimers found in STFs, suggesting it may play multiple roles in signaling. We have carried out a detailed analysis on the role of the Stat1 and Stat2 SH2 domains in the mediation of IFN-α stimulated signals. These studies, which have determined that the SH2 domain of Stat1 and Stat2 can mediate homo- as well as heterodimerization, suggest that a single SH2 domain–phosphotyrosyl interaction is sufficient for dimerization. Moreover, they provide the first direct evidence that the target of the SH2 domain is the STAT tyrosine activation site. In addition, these studies implicate the SH2 domain in another step in the signaling cascade, namely mediation of the interaction between STATs and their activating kinases (i.e. the JAKs).

Keywords: IFN-α/SH2 domains/signal transduction/Stat1/Stat2

Introduction

Cytokines regulate multiple aspects of cell growth through their interactions with a family of related receptors. Characterization of the cytokine family of receptors has led to the recognition that it is composed of several subfamilies. For example, the IL-2 family (e.g. IL-2, IL-4, IL-7, IL-9 and IL-15) all employ a common γC receptor component (Giri *et al.*, 1994; Kishimoto *et al.*, 1994). Similar observations have been made for the IL-3 (e.g. IL-3, IL-5 and GM-CSF) and IL-6 (e.g. IL-6, IL-11, OnM, LIF and CNTF) families (Kishimoto *et al.*, 1994; Schindler and Darnell, 1995). In contrast, the IFN family of receptors (e.g. IFN-α, IFN-γ) are more divergent and only share some distinct structural features (Ho *et al.*, 1993; Schindler and Darnell, 1995). When the cytokine receptors are bound by their corresponding ligands, they become activated and initiate signals directed at both the cytoplasmic and the nuclear compartments of the cell. Many of the nuclear signals culminate in altered patterns of gene expression. It is the unique pattern of genes induced by a ligand (or sub-family of ligands) that is widely believed to play an important role in defining the ensuing biological response.

Until recently, the nature of the molecules that transmit these ligand-specific signals from the receptor to the nucleus has been poorly defined. However, detailed analysis of the ability of interferon (IFN)-α to rapidly induce new genes has led to the identification of a new signaling paradigm, the JAK-STAT pathway (reviewed in Darnell *et al.*, 1994; Schindler and Darnell, 1995). JAK refers to a family of receptor-associated tyrosine kinases, and STAT (Signal Transducers and Activators of Transcription) refers to a family of latent cytoplasmic transcription factors, which are activated by tyrosine phosphorylation. When IFN-α binds its multimeric receptor, two members of the JAK family, Tyk2 and Jak1, are activated. These kinases in turn mediate the activation of Stat1 (also referred to as p91) and Stat2 (also referred to as p113). Once tyrosine-phosphorylated, Stat1 and Stat2 associate into an active Signal Transducing Factor (STF), which translocates to the nucleus (Schindler *et al.*, 1992b). In the nucleus the Stat1:Stat2 complex associates with a DNA binding protein, p48, on the IFN-specific response element (ISRE) and promotes the activation of genes. This active transcription factor is called STF–IFNα or ISGF-3.

Subsequently, many other cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, LIF, OnM, CNTF, Epo, GH, PRL, GM-CSF, G-CSF and IFN-γ have also been shown to signal through this paradigm (Shuai *et al.*, 1992; Bonni *et al.*, 1993; Kontanides and Reich, 1993; Larner *et al.*, 1993; Silvennoinen *et al.*, 1993; Lehmann *et al.*, 1994; Lütticken *et al.*, 1994; Pernis *et al.*, 1994; Rothman *et al.*, 1994; Schindler *et al.*, 1994; Sliva *et al.*, 1994; Azam *et al.*, 1995; Gouilleux *et al.*, 1995; Mui *et al.*, 1995; Schindler and Darnell, 1995; Stahl *et al.*, 1995; K.Shibuya, D.Kaplan and C.Schindler, submitted). For each of these ligands, an STF (e.g. STF–IL2, STF–IL3, STF–IL4, etc.) consisting of either a homo- or heterodimer of one of the seven known STATs has been identified. However, unlike STF–IFNα,

the STAT proteins in these STFs can bind DNA directly (i.e. in the absence of an additional protein). A sequence comparison between Stat2, the IFN-α-specific STAT, and other STATs, reveals an overall homology of 30–50%, with several more highly conserved domains, but fails to identify any obvious difference that might account for the inability of the IFN-α-activated STAT dimer to bind DNA (Fu *et al.*, 1992; Schindler *et al.*, 1992a; Akira *et al.*, 1994; Hou *et al.*, 1994; Wakao *et al.*, 1994; Zhong *et al.*, 1994; Azam *et al.*, 1995; Mui *et al.*, 1995). Notably, one of these conserved domains, the SH2 domain, has been shown to contribute to signal fidelity. Moreover, even small differences in sequence can have important functional consequences (Greenlund *et al.*, 1994, 1995; Hou *et al.*, 1994; Heim *et al.*, 1995; Horvath *et al.*, 1995; Stahl *et al.*, 1995). The most detailed studies on the function of the SH2 domain have been carried out in the IFN-γ system, which signals exclusively through Stat1 (Shuai *et al.*, 1992, 1994). The Stat1 SH2 domain for example, has been shown to play a critical role in the first important step in STAT signaling, receptor recognition. Studies have shown that the Stat1 SH2 domain specifically recognizes Tyr440 of the IFN-γ receptor, which is phosphorylated in response to ligand (Greenlund *et al.*, 1994, 1995; Heim *et al.*, 1995). Once drawn into the dimeric IFN-γ receptor complex, Stat1 becomes activated by tyrosine phosphorylation (Schindler *et al.*, 1992b). Subsequently, by a process that is poorly understood, the activated STATs dissociate from the receptor and homodimerize to form STF–IFNγ. The Stat1 SH2 domain has also been implicated in the dimerization process. For example, studies have shown that the addition of a phosphopeptide representing the activation domain of Stat1, or the addition of Stat1 GST–SH2 fusion protein, will disrupt the Stat1 homodimeric complex (Shuai *et al.*, 1994). Furthermore, since the SH2 domain and activation domains are juxtaposed in Stat1 (and all other STATs), it is likely that they associate through intermolecular rather than intramolecular interactions.

The unique properties of Stat2 (e.g. its divergent carboxy-terminus, its activation by only one receptor, and its reported failure to homodimerize; C.Schindler, unpublished observation; Heim *et al.*, 1995; Schindler and Darnell, 1995) and also the unique nature of the Stat1:Stat2 heterodimer (e.g. its inability to bind DNA directly), prompted us to investigate the potentially divergent role of the Stat1 and Stat2 SH2 domains in mediating IFN-α signal. In a separate report, we provide evidence that the SH2 domain of Stat2 specifically recognizes a phosphotyrosyl residue in the cytoplasmic domain of the IFN-α receptor α-chain (Yan *et al.*, 1996). In this set of studies, we provide evidence that the SH2 domain mediates an interaction with Jak1 and Tyk2, as well as the subsequent STAT homo- or heterodimerization. Biochemical analysis of these interactions has led to a new model of STAT activation.

Results

Stat1 and Stat2 SH2 domains and tyrosine motifs differentially disrupt DNA binding complexes

In our efforts to examine the role of the SH2 domains of Stat1 and Stat2 in IFN-α signaling, a number of reagents including, GST–SH2 fusion proteins, and peptides representing the STAT tyrosine activation domains (potential targets of the SH2 domains), were developed. The 15–20 amino acid (AA) peptides, with a centrally located tyrosine, were prepared either in their native state or as tyrosine-phosphorylated analogs. For the GST–SH2 fusion proteins, wild-type (wt) and mutant (mt) versions were generated. Furthermore, since we were not able to observe any difference between larger GST fusion proteins (e.g. SH3 plus SH2; see Figure 6C) and GST fusion proteins encoding only the SH2 domains (e.g. AA 557–668 for Stat1 and AA 549–672 for Stat2), just the latter were used in these studies. Both the Stat1 and Stat2 GST–SH2 fusion proteins were mutated to help demonstrate specificity for each of the interactions described below. A single point mutation was introduced changing the absolutely conserved arginine in the floor of the SH2 phosphotyrosyl pocket to a lysine. Previous studies have shown that this mutation effectively cripples the ability of SH2 domains to bind phosphotyrosine (Mayer *et al.*, 1992; Waksman *et al.*, 1992). The products of each of these four GST fusion constructs [i.e. wild-type and mutant GST–(Stat1)SH2, and wild-type and mutant GST–(Stat2)SH2] were examined for size and quantity by SDS–PAGE before each study (data not shown).

First, we examined the biological activity of these GST–SH2 domains by assessing their ability to disrupt STAT DNA binding complexes. This was based on the premise that if the SH2 domains mediated dimerization, then exogenously added SH2 domains should compete with the native STAT SH2 domains, thereby disrupting STAT dimers (Shuai *et al.*, 1994). Since the electrophoretic mobility shift assay (EMSA) provides a sensitive assay for dimerized STATs, this assay was used to determine whether the GST–SH2 fusion proteins were biologically active. Consistent with previous studies with a different Stat1 GST–SH2 fusion protein, the addition of our Stat1 GST–SH2 fusion protein led to the disruption of the STF–IFNγ GAS binding complex in a dose-dependent manner, but only after the addition of a mild denaturant (see Figure 1A; Shuai *et al.*, 1994). Pre-treatment of this complex with the denaturant guanidium HCl appears to open the complex sufficiently to allow the GST–SH2 fusion proteins access to their target tyrosine motifs. Moreover, the Stat2 GST–SH2 fusion protein was equally potent in its ability to disrupt STF–IFNγ, indicating that it has an equivalent affinity for the target Stat1 motif. Importantly, neither the GST protein alone, nor the two mutant GST–SH2 fusion proteins, prevented renaturation of the DNA binding complex, demonstrating the specificity of this interaction. A parallel set of studies, adding increasing amounts of the putative tyrosine-phosphorylated targets of these SH2 domains produced a similar result (see Figure 1B). Again, the STF–IFNγ complex was disrupted in a dose-dependent manner by tyrosine-phosphorylated peptides representing the activation domain of Stat1 and Stat2. However, the addition of non-phosphorylated peptide had no effect. These experiments, consistent with previous results (Shuai *et al.*, 1994), suggest that an interaction between the SH2 and tyrosine activation domains of Stat1 mediate homodimerization (i.e. formation of the STF–IFNγ). Moreover, these studies also suggest that the SH2 and tyrosine activation domains of Stat2 recognize the same targets.

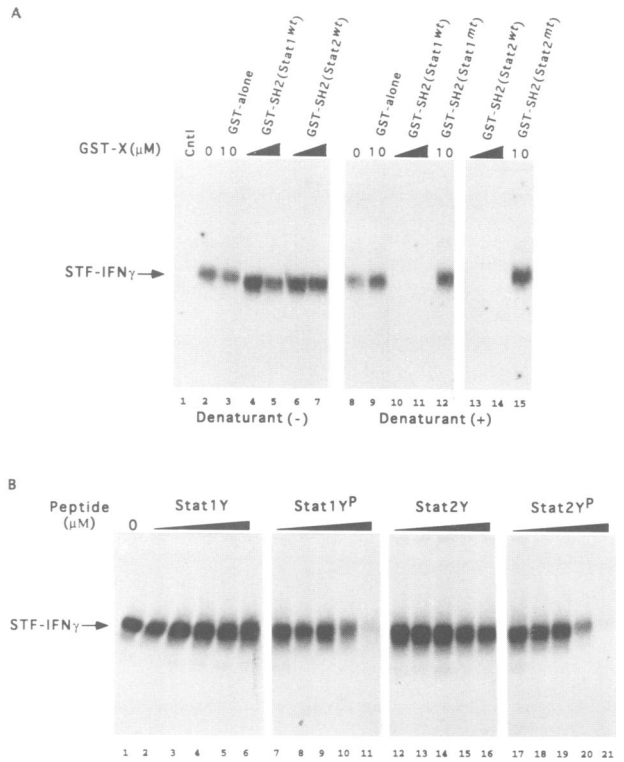

Fig. 1. Stability of STF–IFNγ. (**A**) Disruption of STF–IFNγ by the addition of Stat1 or Stat2 GST–SH2 fusion proteins. IFN-γ stimulated nuclear extracts, prepared from HeLa cells, were incubated with purified preparations of Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins, and assayed by EMSA with an IRF-1 GAS probe as indicated. The GST–SH2 fusion proteins were added at a final concentration of 5 μM (lanes 4, 6, 10 and 13) and 10 μM (lanes 5, 7, 11, 12, 14 and 15). The samples in lanes 8–15 were treated with the denaturant guanidium HCl before addition of the GST fusion proteins. Controls included unstimulated extracts (Cntl), stimulated extract in the absence of any GST fusion protein addition (lanes 2 and 8), and the addition of GST-alone (lanes 3 and 9). (**B**) Disruption of STF–IFNγ by Stat1 or Stat2 peptides. IFN-γ stimulated nuclear extracts, prepared from HeLa cells, were incubated with purified preparations of non-phosphorylated (Stat1Y, Stat2Y) or tyrosine-phosphorylated peptides (Stat1Y^P, Stat2Y^P) representing the activation site of Stat1 and Stat2. Peptides were added at increasing concentrations of 0 μM (lane 1), 5 μM (lanes 2, 7, 12 and 17), 25 μM (lanes 3, 8, 13 and 18), 50 μM (lanes 4, 9, 14 and 19), 100 μM (lanes 5, 10, 15 and 20) and 200 μM (lanes 6, 11, 16 and 21) and assayed as described above.

To provide more definitive evidence that interactions between the SH2 and tyrosine activation domains of Stat1 and Stat2 mediate heterodimerization, a similar set of studies was undertaken on the STF–IFNα (also referred to as ISGF-3; Schindler *et al.*, 1992a,b) DNA binding complexes (see Figure 2). Again, both the wild-type, but not the GST-alone or mutant GST–SH2 fusion proteins, disrupted the complex in a dose-dependent manner. Similarly, the phosphorylated tyrosine motif peptides from Stat1 and Stat2 also disrupted the complex in a dose-dependent manner. In contrast however, the STF–IFNα DNA binding complex was much more sensitive to disruption by these agents. As a matter of fact, pre-treatment with denaturant was not required for disruption by the GST–SH2 fusion proteins. Although these data indicate that the Stat1 and Stat2 GST–SH2 fusion proteins and tyrosine motifs are able to interact specifically with one or more of the protein components of STF–IFNα, they

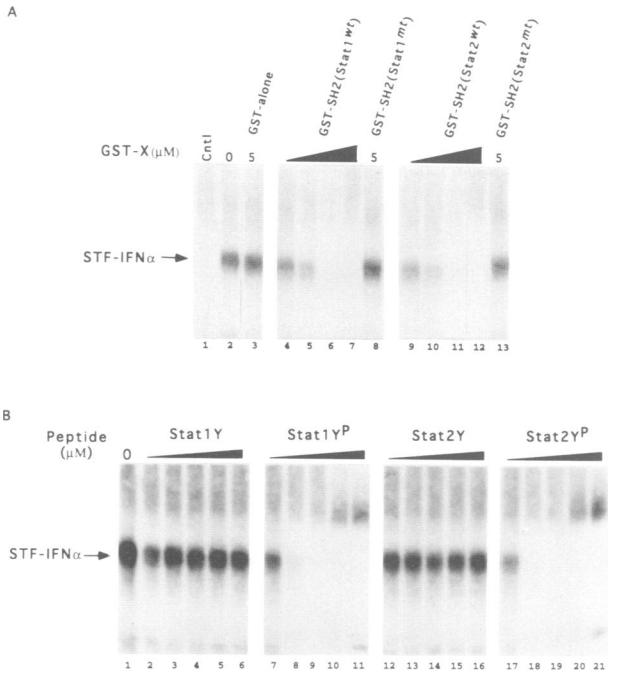

Fig. 2. Stability of STF–IFNα. (**A**) Disruption of STF–IFNα by the addition of Stat1 or Stat2 GST–SH2 fusion proteins. IFN-α stimulated nuclear extracts, prepared from HeLa cells, were incubated with Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins essentially as described in Figure 1, and then assayed by EMSA with an ISG-15 ISRE probe. Final concentrations of added GST fusion proteins were 0.25 μM (lanes 4 and 9), 0.5 μM (lanes 5 and 10), 1.25 μM (lanes 6 and 11) and 5 μM (lanes 3, 7, 8, 12 and 13). Controls are as described in Figure 1 except that GST-alone was added to a final concentration of 5 μM (lane 3). (**B**) Disruption of STF–IFNα by Stat1 and Stat2 peptides. IFN-α stimulated nuclear extracts, prepared from HeLa cells, were incubated with purified preparations of non-phosphorylated (Stat1Y, Stat2Y) or tyrosine-phosphorylated peptides (Stat1Y^P, Stat2Y^P) representing the activation site of Stat1 and Stat2. Peptides were added at increasing concentrations as described in Figure 1B.

also suggest that the STF–IFNα complex is inherently less stable.

Stability of the STF–IFNα and STF–IFNγ DNA binding complexes

Studies with both peptide and GST–SH2 fusion protein competitors indicated that the STF–IFNα DNA binding complex was markedly less stable than the STF–IFNγ DNA binding complex. This was examined directly by the addition of a non-specific denaturant to both DNA binding complexes. As shown in Figure 3A, the STF–IFNα DNA binding complex was at least 250 times more sensitive to the addition of guanidium HCl than the STF–IFNγ DNA binding complex. The unique trimeric structure of STF–IFNα (i.e. Stat1:Stat2 + p48) and the requirement for a non-STAT DNA binding protein (i.e. p48) provided one potential explanation for this observation. Moreover, we reasoned that if Stat1 and Stat2 dimerize through an SH2–phosphotyrosyl interaction, then it should have a stability similar to that observed in Stat1 homodimers (see above). To test this, increasing concentrations of guanidium HCl were added to IFN-α-stimulated extracts, and the complex was then collected by immunoprecipitation. This assay is based on the observation that Stat2 antibodies will immunoprecipitate a

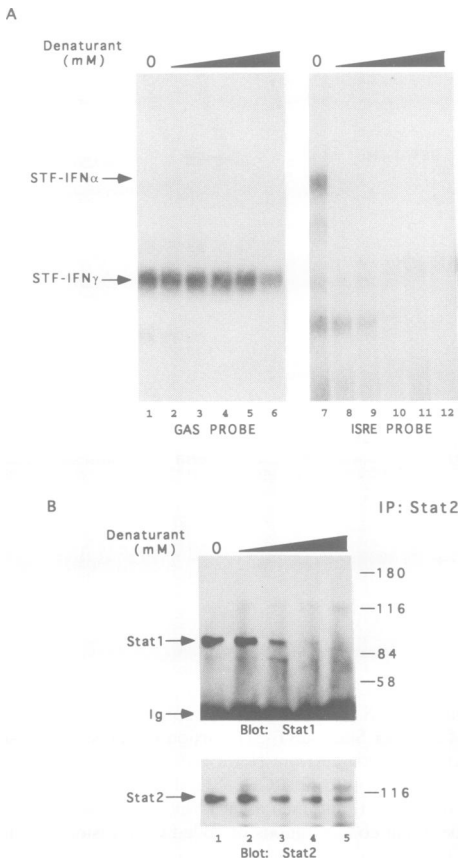

Fig. 3. Comparison of the stability of STF–IFNα and STF–IFNγ. (**A**) Disruption of STF–IFNγ and STF–IFNα by guanidium HCl. STF–IFNγ and STF–IFNα, prepared as described in Figures 1 and 2, were treated with increasing concentrations of guanidium HCl, and then assayed as described in Figures 1 and 2. Final concentrations of added guanidium HCl were, 50 mM (lanes 2 and 8), 100 mM (lanes 3 and 9), 150 mM (lanes 4 and 10), 200 mM (lanes 5 and 11) and 250 mM (lanes 6 and 12). (**B**) Stability of the Stat1:Stat2 dimer. WCE prepared from HeLa cells stimulated with IFN-α alone were treated with increasing concentrations of guanidium HCl, and then immunoprecipitated with a Stat2 antibody. The immunoprecipitated proteins were fractionated by SDS–PAGE and sequentially immunoblotted with a Stat1 and Stat2 antibody as indicated. Final concentrations of the added guanidium HCl were 100 mM (lane 2), 200 mM (lane 3), 250 mM (lane 4) and 300 mM (lane 5). Molecular weight markers (Sigma) were run as indicated in the right margin.

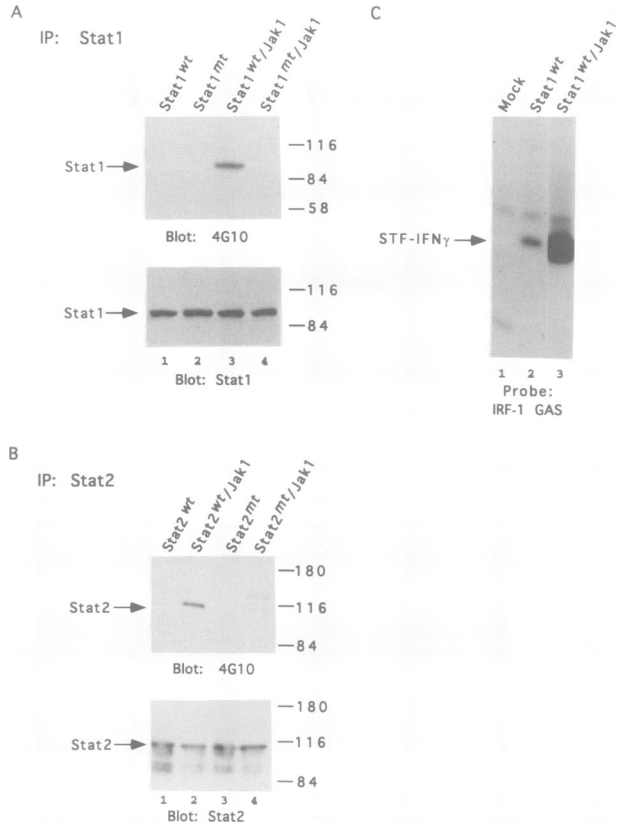

Fig. 4. Activation of recombinant Stat1 and Stat2. (**A**) Immunoblot analysis of recombinant Stat1, prepared from SF-9 cells infected with either a Stat1 (wt or mt) baculovirus (lanes 1 and 2 respectively) or co-infected with Stat1 (wt or mt) and Jak1 baculoviruses (lanes 3 and 4, respectively), after immunoprecipitation with a Stat1 antibody (ab2). Gels were run as described in Figure 3, and then sequentially immunoblotted with antiphosphotyrosine (4G10) or Stat1 antibodies (ab2) as indicated. (**B**) Immunoblot analysis of recombinant Stat2, prepared from SF-9 cells infected with either a Stat2 (wt or mt) baculovirus (lanes 1 and 3 respectively), or co-infected with Stat2 (wt or mt) and the Jak1 baculovirus (lanes 2 and 4 respectively), after immunoprecipitation with a Stat2 antibody. Gels were run as described in Figure 3 and then sequentially immunoblotted with antiphosphotyrosine (4G10) or Stat1 antibodies (ab2) as indicated. (**C**) Analysis of the ability of recombinant Stat1, prepared from singly (lane 2) or doubly (i.e. Stat1/Jak1; lane 3) infected SF-9 cells to bind an IRF-1 GAS probe. Extracts prepared from mock-infected (i.e. vector alone) SF-9 cells are included as a control (lane 1). The mobility of native STF–IFNγ is indicated in the left margin.

Stat1:Stat2 complex after stimulation with IFN-α (Schindler *et al.*, 1992b). As shown in Figure 3B, the ability of activated Stat1 to co-precipitate with activated Stat2 was resistant to the addition of denaturant of up to 200 mM. These studies indicate that the Stat1:Stat2 heterodimer has a stability similar to the Stat1 homodimer (Shuai *et al.*, 1994), and support the notion that a similar dimerization strategy is employed by both of these complexes. However, the requirement for an additional DNA binding protein, p48, in the STF–IFNα DNA binding complex appears to render it inherently less stable to the addition of GST–SH2 fusion proteins, tyrosine motifs, or denaturant, presumably by destabilizing interactions between the Stat1:Stat2 heterodimer, p48, and the ISRE.

Activated Stat1 and Stat2 can be prepared from baculovirus-infected insect cells

The difficulty in carrying out biochemical studies on Stat1 and Stat2 prepared from cellular extracts predicated a recombinant source of these proteins. Hence, recombinant proteins were prepared in insect cells infected with the appropriate baculovirus construct. As shown in Figure 4, infection with a Stat1 recombinant virus yielded generous quantities of the expected 91 kDa Stat1 immunoreactive protein, which became tyrosine-phosphorylated upon co-infection with a recombinant Jak1 baculovirus (Figure 4A). However, a Stat1 baculovirus with the mutation R602K (i.e. SH2 domain), failed to become tyrosine-phosphorylated when co-infected with Jak1 (Figure 4A) or other JAKs (data not shown). Similar observations were made for insect cells infected with baculovirus constructs encoding the wild-type or mutant (i.e. R601K) Stat2 (Figure 4B). The faint ~130 kDa tyrosine-phosphorylated band that co-precipitated with the mutant Stat2 is likely to be Jak1. These data suggest that the STAT

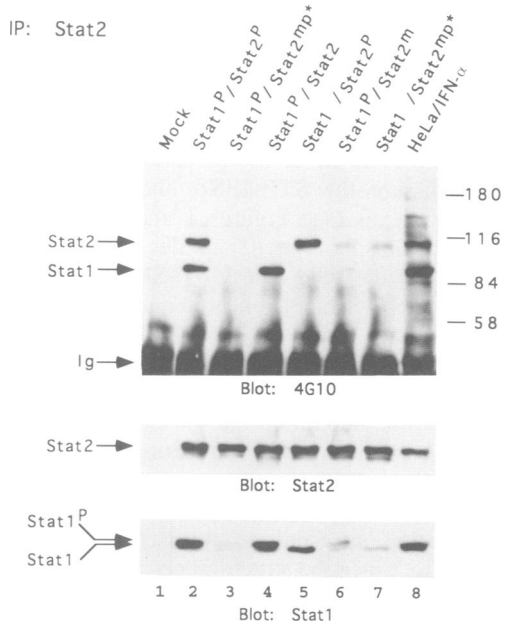

Fig. 5. *In vitro* association of recombinant Stat1 or Stat2. Extracts prepared from singly or doubly infected wt or mt STATs and Jak1 (as indicated) were co-incubated for 20–30 min at 22°C and then analyzed by a Stat2 immunoprecipitation assay, which was carried out at 4°C. Immunoprecipitates were fractionated and sequentially immunoblotted with an antiphosphotyrosine, Stat2, and Stat1 (ab2) recognizing antibodies as described in Figures 3 and 4. Extracts prepared from mock-infected (i.e. vector alone) SF-9 cells are included as a control (lane 1). Extracts included in the experiment were: Stat1 (singly infected with Stat1 baculovirus), Stat2 (singly infected with Stat2 baculovirus), $Stat2^{m}$ (singly infected with mutant R601K Stat2 baculovirus), $Stat1^{P}$ (co-infection with Stat1 and Jak1 viruses), $Stat2^{P}$ (co-infection with Stat2 and Jak1 viruses), $Stat2^{mp*}$ (co-infected with mutant R601K Stat2 and Jak1 viruses). Immunoprecipitates of bona fide Stat1:Stat2 heterodimers prepared from IFN-α-stimulated HeLa cells are shown as a control (lane 8).

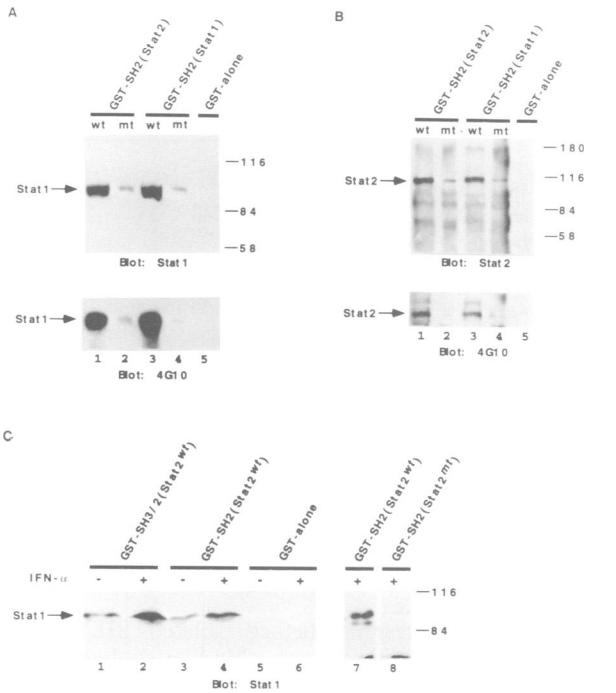

Fig. 6. STAT GST–SH2 fusion proteins specifically bind activated Stat1 and Stat2. (**A**) GST–SH2 pull downs of recombinant Stat1. Immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins were incubated with activated recombinant Stat1 (i.e. $Stat1^{P}$), as indicated. Bound proteins were eluted, fractionated and sequentially immunoblotted with Stat1 and antiphosphotyrosine antibodies as described in Figures 3 and 4. Binding to GST-alone (lane 5) is shown as a control. (**B**) GST–SH2 pull downs of recombinant Stat2. Immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins were incubated with activated recombinant Stat2 (i.e. $Stat2^{P}$), as indicated. Bound proteins were eluted, fractionated and sequentially immunoblotted with Stat2 and antiphosphotyrosine antibodies as described in Figures 3 and 4. Binding to GST-alone (lane 5) is shown as a control. (**C**) GST–SH2(Stat2) pull downs of Stat1 from IFN-stimulated MEL cells. WCE were prepared from unstimulated (–) or IFN-α- (30 min) stimulated (+) MEL cells, and then incubated with wt or mt GST–SH2 or GST–SH3+SH2 (GST–SH3/2) fusion proteins as indicated. Bound proteins were detected by immunoblotting with Stat1 antibody as described in Figure 3. Binding to GST-alone (lanes 5 and 6) is shown as a control.

SH2 domains may play an essential role in JAK-mediated STAT activation (i.e. tyrosine phosphorylation).

Next, the biological activity of the recombinant activated Stat1 was examined by a DNA binding assay. As shown in Figure 4C, Stat1-only infected insect cells yielded a low level of DNA binding activity, which correlated with a low level of tyrosine phosphorylation (data not shown). In contrast, lysates prepared from cells co-infected with Stat1 and Jak1, exhibited a profound increase in DNA binding activity. The extent of this DNA binding activity, which co-migrated with bona fide STF–IFNγ (Pine *et al.*, 1994; Rothman *et al.*, 1994), correlated well with the extent of tyrosine phosphorylation (data not shown), suggesting that a majority of the recombinant Stat1 was biologically active. However, examination of the biological activity of recombinant Stat2 was more difficult, since activated Stat2 is not known to bind DNA directly. We took advantage of the ability of a Stat2 antibody to immunoprecipitate the Stat1:Stat2 heterodimer, reasoning that inactive Stat2 would be unable to participate in complex formation. As shown in Figure 5, the Stat2 antibody was able to co-immunoprecipitate $Stat1^{P}$ (i.e. tyrosine-phosphorylated) from a mixture of recombinant $Stat1^{P}$ and recombinant $Stat2^{P}$. Next, in an effort to determine whether both STAT partners needed to be phosphorylated (i.e. activated) to form a stable complex, extracts from singly (i.e. Stat1 or Stat2 only) and doubly (i.e. Stat1 or Stat2 plus Jak1) infected insect cells were co-incubated. Surprisingly, unphosphorylated Stat1 was co-immunoprecipitated as effectively as $Stat1^{P}$ (compare lanes 2 and 5) with the Stat2 antibody. Likewise, unphosphorylated Stat2 co-immunoprecipitated $Stat1^{P}$ as effectively as did $Stat2^{P}$ (compare lanes 2 and 4). Moreover, studies with the mutant Stat2 (R601K) demonstrated that the ability to co-immunoprecipitate Stat1 was dependent on the wild-type Stat2 SH2 domain (lanes 3, 6 and 7). In addition to providing support for the role of SH2 domains and STAT phosphotyrosyl residues in mediating Stat1:Stat2 heterodimerization, these studies indicate that only one partner needs to be phosphorylated to form a stable complex. These observations are consistent with *in vivo* observations (Schindler *et al.*, 1992a; Zhang *et al.*, 1995; see Discussion for details).

Activated Stat1 and Stat2 bind to GST–SH2 fusion proteins

With an available source of recombinant Stat1 and Stat2 proteins and the GST–SH2 fusion proteins, it was possible

Table I. Stat1 and Stat2 phosphopeptides bind Stat1 and Stat2 SH2 domains *in vitro*

GST fusion proteins	Bound phosphopeptides (c.p.m. ± SD)		
	Stat1•Y^P	Stat2•Y^P	CS-3C•Y^P
GST–SH2($Stat1^{wt}$)	1429 ± 18	1243 ± 25	56 ± 4
GST–SH2($Stat1^{mt}$)	77 ± 6	63 ± 4	51 ± 3
GST–SH2($Stat2^{wt}$)	1271 ± 24	1301 ± 29	52 ± 5
GST–SH2($Stat2^{mt}$)	76 ± 4	85 ± 5	50 ± 5
GST-alone	52 ± 3	53 ± 4	49 ± 4

Synthetic peptides (Research Genetics) representing the tyrosine activation site of Stat1 (Stat1•Y^P) or the tyrosine activation site of Stat2 (Stat2•Y^P) or an irrelevant peptide (CS-3C•Y^P), were tyrosine-phosphorylated *in vitro* with [γ-^{32}P]ATP and incubated with immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins as indicated. After extensive washing, the peptides were eluted and the recovered ^{32}P determined by scintillation counting. An irrelevant tyrosine-phosphorylated peptide (CS-C3) and a GST-alone fusion protein were included as controls. The results represent the average of three independent experiments.

to determine directly whether isolated SH2 domains mediated dimerization. *In vitro* binding studies, between activated recombinant STATs and the four GST–SH2 fusion proteins were carried out. Consistent with the preceding results, the wild-type, but not mutant, Stat1 and Stat2 GST–SH2 fusion proteins bound activated (i.e. tyrosine-phosphorylated) recombinant Stat1 (see Figure 6A). Likewise, wild-type, but not mutant, Stat1 and Stat2 GST–SH2 fusion proteins bound activated recombinant Stat2 (see Figure 6B). These studies demonstrate that the isolated SH2 domains from Stat1 and Stat2 mediate association with activated (i.e. tyrosine-phosphorylated) Stat1 and Stat2. Furthermore, they suggest that the SH2 domains of both Stat1 and Stat2 can mediate homo- as well as heterodimerization.

The ability of the GST–SH2 fusion proteins to bind to significant quantities of recombinant STATs suggested that they might also be able to bind bona fide STATs. To test this possibility, extracts were prepared from control and IFN-α-stimulated MEL cells (Ralph *et al.*, 1995). Both a GST–(Stat2)SH3/SH2 and a GST–(Stat2)SH2, but not a GST-alone fusion protein, were able to bind activated native Stat1 (see Figure 6C). The low level of Stat1 binding observed in the unstimulated cells can be attributed to a low level of Stat1 tyrosine phosphorylation observed in MEL cells after a standard overnight pre-treatment with IFN-γ (to boost the level of Stat1; Schindler *et al.*, 1992b). Again, when wild-type and mutant GST–SH2 fusion proteins were examined, only the wild-type Stat2 GST–SH2 fusion protein was able to bind to native activated Stat1 (see Figure 6C). The MEL cell studies also implicate the SH2 domain in dimerization and confirm the results of the more extensive studies carried out with the recombinant STATs.

Stat1 and Stat2 tyrosine motifs specifically bind to GST–SH2 fusion proteins

The studies in the preceding section demonstrated the role of the SH2 domain in mediating STAT dimerization. In an effort to obtain complementary data with the presumed phosphotyrosyl substrates of these SH2 domains, and to demonstrate directly that they are targets of these SH2 domains, peptide binding studies were carried out. In these studies, peptides representing the activation domain of Stat1, Stat2 and an irrelevant peptide (CS-C3) were phosphorylated *in vitro* with [γ-^{32}P]ATP and incubated with all four GST fusion proteins. The Stat1 and Stat2 peptides were identical to the preparations that had been shown to disrupt the STF–IFNα and STF–IFNγ DNA binding complexes (see Figures 1 and 2). As shown in Table I, the phosphorylated peptides were significantly bound only by the wild-type GST–SH2 fusion proteins. The mutant GST–SH2 fusion proteins had the same low level of binding activity observed with the irrelevant peptide and the GST-alone protein. These studies provide the first direct evidence that the SH2 domain of Stat1 will bind the tyrosine activation site of either Stat1 or Stat2. Furthermore, consistent with the recombinant STAT binding studies, these experiments suggest that the Stat2 SH2 domain can mediate both a reciprocal interaction with the Stat1 activation site and an interaction with its own activation site. Since the structural organization of Stat2 is unlikely to permit an intramolecular SH2–phosphotyrosine interaction, these observations suggest that Stat2 homodimers may exist.

Stat1 and Stat2 GST–SH2 fusion proteins bind JAK kinases

In the last set of studies, we were interested in following up on the observation that a point mutation in the SH2 domains of both Stat1 and Stat2 rendered the proteins incapable of being tyrosine-phosphorylated (see Figure 4). The dramatic effect of this conservative mutation suggested that the STAT SH2 domain might mediate a direct and obligate interaction with the JAKs. The availability of recombinant Jak1, Tyk2 and the GST–SH2 fusion proteins provided an opportunity to test this. Recombinant Jak1 or Tyk2, prepared from singly infected insect cells, was incubated with wild-type and mutant GST–SH2 fusion proteins. Both of the wild-type, but not mutant, Stat1 and Stat2 GST–SH2 fusion proteins were able to bind recombinant Jak1 and Tyk2 (see Figure 7A and B), suggesting that these domains mediate a specific, and direct interaction with a phosphotyrosyl residue in the kinases. Since recombinant JAK kinases prepared from insect cells are autophosphorylated and biologically active (see above and Colamonici *et al.*, 1994; Quelle *et al.*, 1995), they certainly contain the phosphotyrosyl residues necessary for biological activity, but could also contain additional non-physiological phosphotyrosines. In an effort to demonstrate that phosphotyrosyl residues which are found in physiologically activated JAKs also interact with the SH2 domains of Stat1 and Stat2, the experiment was repeated with extracts prepared from IFN-α-stimulated cells. Again, these studies indicate that wild-type, but not mutant, Stat1 and Stat2 GST–SH2 fusion proteins are able to bind activated Tyk2 (see Figure 7C). In a related set of studies, we have also been able to co-immunoprecipitate Stat1 with either a Tyk2 or Jak1 antibody from IFN-stimulated cellular extracts (data not shown). These studies, along with the compelling results from insect cells co-infected with mutant Stat1 and Stat2, suggest that the SH2 domains mediate an obligate interaction with the JAK kinases. Confirmation of this model will require the identification of the STAT SH2 binding site on the JAKs.

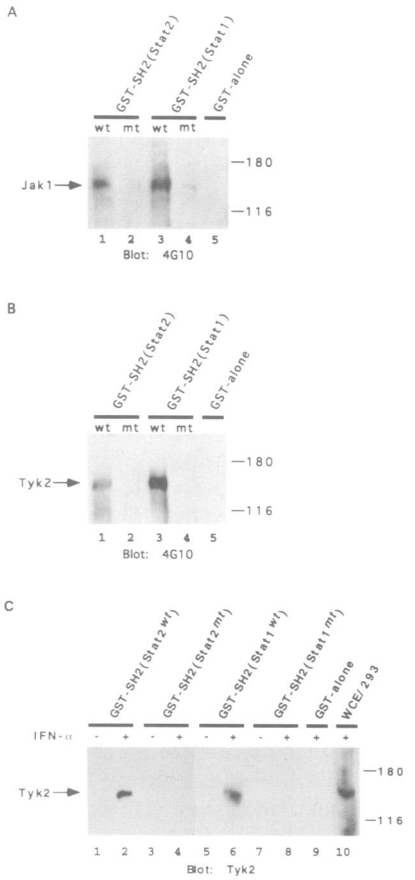

Fig. 7. STAT GST–SH2 fusion proteins specifically bind activated Jak1 and Tyk2. (**A**) GST–SH2 pull downs of recombinant Jak1. Immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins were incubated with activated recombinant Jak1, eluted and analyzed by immunoblotting with antiphosphotyrosine antibodies as described in Figures 4 and 6. Binding to GST-alone (lane 5) is shown as a control. (**B**) GST–SH2 pull downs of recombinant Tyk2. Immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins were incubated with activated recombinant Tyk2, eluted and analysed by immunoblotting with antiphosphotyrosine antibodies as described in Figures 4 and 6. Binding to GST-alone (lane 5) is shown as a control. (**C**) GST–SH2 pull downs of natively activated Tyk2. WCE, prepared from unstimulated (–) or IFN-α- (15 min) stimulated (+) 293 cells, were incubated with immobilized Stat1 (wt or mt) or Stat2 (wt or mt) GST–SH2 fusion proteins as indicated. Bound proteins were eluted, fractionated and immunoblotted with Tyk2 antibodies (as recommended; Signal Transduction Labs). Binding to GST-alone is indicated in lane 9, and the mobility of native Tyk2 (i.e. in 293 WCE) is shown in lane 10.

Discussion

The characterization of signaling pathways initiated by both tyrosine kinase receptors and cytokine receptors has highlighted the important role SH2 domains play in mediating signal transduction (Hunter, 1995). The ability of SH2 domains to recognize phosphotyrosines in the context of a specific 4–5 amino acid motif (i.e. a tyrosine motif), engenders them with the ability to mediate interactions with specific target proteins (Songyang *et al.*, 1993; Larose *et al.*, 1995). Consistent with their high degree of conservation, the SH2 domains found in STAT proteins also appear to be important in regulating the fidelity of STAT signal transduction (Schindler and Darnell, 1995). Recent studies have shown that these SH2 domains recognize specific activated tyrosine motifs found in the cytoplasmic domain of the appropriate cytokine receptors (Greenlund *et al.*, 1994; Hou *et al.*, 1994; Heim *et al.*, 1995; Stahl *et al.*, 1995; Yan *et al.*, 1996). Other studies have provided compelling, albeit indirect, evidence that SH2 domains also mediate the subsequent homodimerization of Stat1 in response to IFN-γ (Shuai *et al.*, 1994). We now present direct evidence that homo- as well as heterodimerization of STATs is mediated by the interaction between the SH2 domain of one STAT and the tyrosine activation site of the other STAT. Moreover, we demonstrate that a single SH2–phosphotyrosyl interaction is sufficient for dimerization. Additionally, we present evidence that the SH2 domain may play an obligatory role in the interaction between the STATs and the activating JAK kinases.

Studies on the IFN-α system, where the STAT proteins were first identified (Schindler *et al.*, 1992a,b), have also led to the identification of a tyrosine motif on the cytoplasmic domain of the IFN-α receptor α-chain (IFNAR). This motif appears to mediate a specific interaction with the SH2 domain of Stat2, enabling recruitment to the receptor (Yan *et al.*, 1996). Stat1 is then drawn into the receptor complex, perhaps through an interaction with activated Stat2 (see below; Leung *et al.*, 1995; Yan *et al.*, 1996), and also activated by a Jak1/Tyk2-dependent event. By an unknown mechanism, the activated Stat1 and Stat2 dissociate from the receptor, and as a complex (i.e. Stat1:Stat2) translocate to the nucleus, where they mediate transcriptional activation. However, unlike all of the other STAT pathways characterized to date, the Stat1:Stat2 heterodimer has no intrinsic DNA binding activity and must interact with additional DNA binding protein to form a transcriptionally active complex (Kessler *et al.*, 1990; Darnell *et al.*, 1994). Moreover, other unique properties of Stat2 (e.g. its species-specific carboxy-terminus, its activation by only one receptor, its reported failure to homodimerize) (unpublished observations; Heim *et al.*, 1995; Schindler and Darnell, 1995), suggested that its SH2 domain might provide one of the more divergent examples of STAT SH2 domains.

In an effort to examine more precisely the role of the SH2 domain in mediating signaling events in the IFN-α system, a number of reagents were developed. This included peptides (both tyrosine-phosphorylated and non-phosphorylated) representing the tyrosine activation domains of the Stat1 and Stat2, and GST–SH2 fusion proteins encoding the SH2 domains of Stat1 and Stat2 (wild-type as well as mutated). Conservatively mutated SH2 domains were included to demonstrate specificity. Also, although larger fusion proteins were initially prepared (e.g. SH3 + SH2 + carboxy-terminus, or SH3 + SH2), the SH2 'only' fusion proteins were used in all of the studies since they were able to mediate all of the interactions examined (see Figure 6). For example, GST–SH2 fusion proteins, and the phosphopeptides, were able to disrupt specifically the STF–IFNα and STF–IFNγ DNA binding complexes, implicating both components in complex formation. A more direct set of binding studies, carried out with the GST–SH2 fusion proteins and recombinant Stat1 and Stat2, demonstrated that the SH2 domains of Stat1 and Stat2 are equivalent in their abilities to mediate homo- and heterodimerization of STATs. More importantly, a parallel set with the tyrosine activation

domain peptides, demonstrated that the Stat1 and Stat2 SH2 domains specifically recognize the activation sites of Stat1 and Stat2, providing critical support for this model of STAT dimerization.

During the course of these studies several additional intriguing properties of the IFN signaling pathway were noted. First, it became apparent that the DNA binding complex activated by IFN-α, STF–IFNα, was inherently less stable than the DNA binding complex activated by IFN-γ, STF–IFNγ. Our studies demonstrate that this increased instability is likely to be due to the trimeric structure of the DNA binding complex (e.g. Stat1:Stat2 + p48), since the stability of Stat1:Stat2 heterodimer is equivalent to the Stat1:Stat1 homodimer. Although these studies provide little physiological insight into the significance of this instability, it may correlate with the more transient nature of STF–IFNα:ISRE transcriptional responses (Decker *et al.*, 1989). Second, we were able to demonstrate that a single SH2-phosphotyrosyl interaction is sufficient for Stat1:Stat2 heterodimerization. This unanticipated result is actually consistent with several *in vivo* observations. For example, during the purification of the STF–IFNα DNA binding complex (Schindler *et al.*, 1992a), stoichiometrically equal quantities of phosphorylated (i.e. on tyrosine) and non-phosphorylated Stat1 were co-purified (C.Schindler, unpublished observation), indicating that the non-phosphorylated Stat1 was incorporated into the Stat1:Stat2 heterodimers isolated from HeLa cells. More recently, a similar observation has been made with the Stat1:Stat3 heterodimer activated by IL-6 stimulation. We have determined that early after activation, only the Stat3 component of this heterodimer is tyrosine-phosphorylated, and able to mediate the observed dimerization (Zhang *et al.*, 1995). Additionally, the ability to dimerize through a single phosphotyrosine would be critical for the STAT activation model proposed by Leung and co-workers (Leung *et al.*, 1995; Yan *et al.*, 1996). In this model, the recruitment of Stat1 to the receptor complex in response to IFN-α is mediated by an interaction between the SH2 domain of Stat1 and the phosphotyrosine of activated Stat2. Third, we found evidence that Stat2 can form stable homodimers. More recently, we have been able to demonstrate that these homodimers bind DNA, suggesting they may be transcriptionally active (unpublished observation).

However, our most intriguing observation was the inability of an SH2 mutant of Stat1 and Stat2 to become tyrosine-phosphorylated in insect cells. The conservative nature of the mutation, indicated that the JAK-mediated activation of STATs is likely to require a direct interaction between the SH2 domain of the target STAT and a phosphotyrosyl residue of the activating JAK. Interestingly, it has recently been suggested that SH2-mediated interactions between a substrate and tyrosine kinase may be very common (Songyang *et al.*, 1995). We therefore carried out a set of binding studies and determined that the SH2 domains of Stat1 and Stat2 can specifically bind both recombinant, as well as native activated Jak1 and Tyk2. Moreover, the GST–SH2 fusion proteins specifically bind an amino-terminally truncated isoform of Tyk2, which appears to be a natural degradation product (data not shown). Not only does this observation localize the STAT interacting domain to a region that is distinct from the domain that mediates interaction with the receptor (i.e. the amino-terminus; Barbieri *et al.*, 1994; Velazquez *et al.*, 1995; Zhao *et al.*, 1995), but it implies that this interaction must occur independently of the receptor. This finding is consistent with the recent demonstration that Stat5 and Jak3 specifically interact (Migone *et al.*, 1995; K.Shibuya,D.Kaplan and C.Schindler, submitted).

Our studies on the SH2 domains of STATs suggest that they are likely to mediate several steps in signal transduction. Although the observation that SH2 domains may mediate a specific interaction between JAKs and STATs necessitates a modification of the current model of JAK-STAT signaling (Greenlund *et al.*, 1995; Schindler and Darnell, 1995), it provides a potential solution for the conundrum: what drives the STATs (i.e. SH2 domains) to dissociate from a higher-affinity interaction with the receptor phosphotyrosyl, to form a dimer whose association is mediated by a lower-affinity phosphotyrosyl interaction (Greenlund *et al.*, 1995), especially if only a single SH2-phosphotyrosyl interaction is required for dimerization? We propose a sequential model in which, after binding to the receptor tyrosine motif, the SH2 domain shifts its target to a tyrosine motif in the kinase itself. This interaction with the kinase may be stabilized by additional domains (data not shown). Activation of the STATs would then destabilize the interaction with the kinase, and the STATs would be driven to form energetically favorable dimers. Interestingly, *in vitro* binding studies suggest that the avidity of STATs for receptor tyrosine motifs (e.g. the IFN-α and IFN-γ receptors) is significantly decreased once the STATs are tyrosine phosphorylated (Yan *et al.*, 1996). Testing this revised model of JAK-STAT signaling will be the basis for further studies on IFN signal transduction.

Materials and methods

Cell culture

Cell culture reagents were purchased from Gibco. SF-9 insect cell culture and infection with baculoviruses were carried out as described (Colamonici *et al.*, 1994; Yan *et al.*, 1996). HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 1% fetal calf serum (FCS), 5% calf serum and 2 mM L-glutamine. The melanoma cell line SK-MEL-28 (ATCC) was grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and sodium pyruvate (Ralph *et al.*, 1995). 293 cells were grown in DMEM supplemented with 10% FCS and 2 mM L-glutamine. All cell types were stimulated for 15 min or as indicated with 1000 U/ml IFN-α (Genentech) after a 16 h pre-incubation in 1 ng/ml of IFN-γ (Hoffmann-La Roche) unless otherwise indicated. Short IFN-γ stimulations (i.e. 15 min) were carried out with 5 ng/ml of IFN-γ.

Cellular extracts and DNA binding assays

Cytoplasmic, nuclear, WCE (whole cell extracts) and shift probes were prepared as previously described (Pine *et al.*, 1994; Rothman *et al.*, 1994). Recombinant STATs and JAKs were prepared from insect cells infected with Stat1 (wild-type and mutant), Stat2 (wild-type and mutant), Jak1 and Tyk2 baculovirus constructs in 1× lysis buffer (see below) as previously described (Colamonici *et al.*, 1994; Yan *et al.*, 1996). All shift reactions were done with the ISG15 ISRE probe gatcTTTCACTTT-CTAGTTTCACTTTCCCTTT (Levy *et al.*, 1989), or the IRF1-GAS probe, gatcGATTTCCCCGAAAT (Oligos Etc.) as previously described (Pine *et al.*, 1994; Rothman *et al.*, 1994), with the following modifications. GST fusion proteins (5.5 μl) buffered in 50 mM Tris, pH 8.0, or peptides (1 μl) buffered in 25 mM Tris, pH 7.4, were added to extracts (1–2 μl) for 15 min at 22°C before the standard shift binding reaction (final volume, 12.5 μl). Denaturation with guanidium hydrochloride was carried out as previously described (Shuai *et al.*, 1994).

Preparation of GST fusion proteins and recombinant STATs

STAT cDNA fragments were cloned into pGEX vectors and expressed in bacteria (Schindler *et al.*, 1992a; Frangioni and Neel, 1993). Briefly, for the GST–Stat1 fusion proteins, a fragment encoding AA 557–668 (SH2) or AA 490–668 (SH3/SH2) was cloned, in-frame, into the *Nco*I and *Xho*I sites of pGEX-KG. The point mutant Arg602 to Lys602 was generated by altering codon 602 from CGG to AAG by site-directed mutagenesis (Clontech) (Deng and Nickloff, 1992). Mutagenesis was confirmed by sequencing the entire pGEX-KG-Stat1-SH2 insert (Amersham/USB). The GST–Stat2 fusion proteins were created by cloning a fragment encoding AA 549–672 (SH2) or 499–672 (SH3/SH2), in-frame, into the *Bam*HI site of pGEX-2T or *Eco*RI site of pGEX-3X, respectively. The point mutant Arg601 to Lys601 was generated by altering codon 601 from CGC to AAG and then confirmed as described above for Stat1. The recombinant STATs were created by cloning the coding regions of the human Stat1 and Stat2 cDNAs (Fu *et al.*, 1992; Schindler *et al.*, 1992a) into the baculovirus shuttle vector pVL1393 (Invitrogen). Recombinant STAT baculovirus constructs were rescued from SF-9 cells after co-transfection with the appropriate shuttle vector and a mutant baculovirus (BaculoGold; Pharmigen). Mutant recombinant STATs were obtained by substituting a restriction fragment isolated from the mutant GST–SH2(Stat1) (i.e. a *Hind*III–*Sma*I fragment) or GST–SH2(Stat2) (i.e. a *Stu*I–*Hind*III fragment) constructs into the appropriate STAT shuttle vector.

Purification of the GST fusion proteins was carried out as described by Frangioni and Neel (1993). Purified proteins were recovered from the glutathione (GSH)–agarose beads (Sigma) by elution with 10 mM GSH (Gibco), 100 mM Tris–HCl, pH 9.6, 150 mM NaCl, 5 mM DTT and 0.1% Triton X-100. After dialysis into PBS, adjusted to 1% Triton X-100, the concentration of each fusion protein was determined by SDS–PAGE/Coomassie staining. Subsequently, the fusion proteins were recoupled to GSH–agarose at ~300 μg of protein per 50 μl of the beads (24 h at 4°C). After extensive washing with PBS/1% Triton X-100, the beads were equilibrated in 1× lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.5% BSA) in preparation for binding studies (see below).

Protein binding studies

Binding studies with recombinant and native STATs and JAKs were carried out by incubating the cell lysates (2–3 mg total cell proteins) with 150 μg (~50 μl) of immobilized GST fusion proteins in 400 μl of 1× lysis buffer for 4 h at 4°C with constant agitation. The complexes were extensively washed with 1× lysis buffer. The bound proteins were eluted from the beads by heating them to 95°C in sample buffer, and then fractionated on a 7% SDS–polyacrylamide gel. The gel was transferred to nitrocellulose (Schleicher and Schuell) and immunoblotted with Stat1 (ab1 or ab2; Rothman *et al.*, 1994), Stat2 (recognizing amino acids 713–751), Tyk2 (Transduction Laboratories) and antiphosphotyrosine (4G10; Upstate Biological Inc.) antibodies as previously described (Pine *et al.*, 1994). Bands were detected by ECL as specified by the manufacturer (Amersham). The immunoprecipitations were performed with a Stat1 antibody (ab2) or Stat2 antibody as previously described (Schindler *et al.*, 1992b).

Peptide binding studies

Synthetic peptides (Research Genetics Inc.) were labeled with [γ-^{32}P]ATP to a specific activity of 2500 c.p.m./μg of peptide with purified p56^{lck} kinase as recommended by the manufacturer (Upstate Biotechnology Inc.) and isolated by centrifugation through membranes with MW exclusions of 10 kDa (Centricon). The peptides represent amino acids 693–712 of Stat1; amino acids 684–698 of Stat2; and for the irrelevant peptide CS-3C (a generous gift of C.Saris) they are amino acids 623–637 of the IL-3 receptor AIC2A (Gorman *et al.*, 1990). Binding studies were carried out with 2 μg of ^{32}P-labeled peptides and 30 μg of immobilized GST–SH2 fusion proteins in 100 μl of PBS, 1% Triton X-100 for 2 h at 4°C. After the binding reaction, the beads were washed four times with 1 ml of cold binding buffer, and the bound peptides were then eluted by boiling in SDS–PAGE sample buffer. The recovered ^{32}P was determined by scintillation (cocktail from ICN) counting (1211 Rackbeta; LKB). Each binding study was repeated three times.

Acknowledgements

We would like to thank Dan Lazner for technical support. We would also like to thank Dr Robert Schreiber for making his wild-type GST–SH2(Stat1) fusion constructs available for mutagenesis and Dr P.Sorter (Hoffmann-La Roche) for providing human IFNs. This work was supported by NIH Grant HL 21006-17 (C.S.) and CA 56862 (J.K.), the James S.McDonnell Foundation (C.S. and S.G.), and Berlex Laboratories (C.S.). J.K. is a recipient of an American Cancer Society Junior Faculty Award and an Irma T.Hirschl Scholar.

References

Akira,S., Nishio,Y., Inoue,M., Wang,X.-J., Wei,S., Matsusaka,T., Yoshida,K., Sudo,T., Naruto,M. and Kishimoto,T. (1994) Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell*, **77**, 63–71.

Azam,M., Erdjument-Bromage,H., Kreider,B.L., Xia,M., Quelle,F., Basu,R., Saris,C., Tempst,P., Ihle,J.N. and Schindler,C. (1995) Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J.*, **14**, 1402–1411.

Barbieri,G.L., Velazquez,L., Scrobogna,M., Fellous,M. and Pellegrini,S. (1994) Activation of the protein tyrosine kinase tyk2 by interferon α/β. *Eur. J. Biochem.*, **223**, 427–435.

Bonni,A., Frank,D.A., Schindler,C. and Greenberg,M.E. (1993) Characterization of a pathway for Ciliary Neurotropic Factor signaling to the nucleus. *Science*, **262**, 1575–1579.

Colamonici,O.R., Yan,H., Domanski,P., Handa,R., Smalley,D., Mullersman,J., Witte,M., Krishnan,K. and Krolewski,J. (1994) Direct binding and tyrosine phosphorylation of the α-subunit of the type I IFN receptor by the p135-tyk2 tyrosine kinase. *Mol. Cell. Biol.*, **14**, 8133–8142.

Darnell,J.E., Kerr,I.M. and Stark,G.M. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415–1421.

Decker,T., Lew,D.J., Cheng,Y.-S., Levy,D.E. and Darnell,J.E. (1989) Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. *EMBO J.*, **8**, 2009–2014.

Deng,W.P. and Nickloff,J.A. (1992) Site directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.*, **200**, 81–88.

Frangioni,J.V. and Neel,B.G. (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.*, **210**, 179–187.

Fu,X., Schindler,C., Improta,T., Aebersold,R. and Darnell,J.E. (1992) The proteins of ISGF-3, the IFN-α induced transcription activator, define a new family of signal transducers. *Proc. Natl Acad. Sci. USA*, **89**, 7840–7843.

Giri,J.G., Ahdieh,M., Eisenman,J., Shanebeck,K., Grabstein,K., Kumaki,S., Namen,A., Park,L.S., Cosman,D. and Anderson,D. (1994) Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.*, **13**, 2822–2830.

Gorman,D.M., Itoh,N., Kitamura,T., Schreurs,J., Yonehara,S., Yahara,I., Arai,K. and Miyajima,A. (1990) Cloning and expression of a gene encoding an interleukin 3 receptor-like protein: Identification of another member of the cytokine receptor gene family. *Proc. Natl Acad. Sci. USA*, **87**, 5459–5463.

Gouilleux,F., Pallard,C., Dusanter-Fourt,I., Wakao,H., Haldosen,L.-A., Norstedt,G., Levy,D. and Groner,B. (1995) Prolactin, growth hormone, erythropoeitin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *EMBO J.*, **14**, 2005–2013.

Greenlund,A., Farrar,M., Viviano,B. and Schreiber,R. (1994) Ligand-induced IFN-γ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J.*, **13**, 1591–1600.

Greenlund,A., Morales,M.O., Viviano,B.L., Yan,H., Krolewski,J. and Schreiber,R.D. (1995) STAT recruitment by tyrosine phsophorylated receptors: an ordered, reversible affinity driven process. *Immunity*, **2**, 677–687.

Heim,M.H., Kerr,I.M., Stark,G.R. and Darnell,J.E. (1995) Contribution of STAT SH2 groups to specific interferon signaling by the JAK-STAT pathway. *Science*, **267**, 1347–1349.

Ho,A.S., Liu,Y., Khan,T.A., Hsu,D.H., Bazan,J.F. and Moore,K.W. (1993) A receptor for interleukin 10 is related to interferon receptors. *Proc. Natl Acad. Sci. USA*, **90**, 11267–11271.

Horvath,C.M., Wen,Z. and Darnell,J.E. (1995) A STAT protein domain that determines DNA sequence recognition suggests a novel DNA binding domain. *Genes Dev.*, **9**, 984–994.

Hou,J., Schindler,U., Henzel,W.J., Ho,T.C., Brasseur,M. and McKnight,S.L. (1994) An Interleukin-4 induced transcription factor: IL-4 Stat. *Science*, **265**, 1701–1705.

Hunter,T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell*, **80**, 225–236.

Kessler,D.S., Veals,S.A., Fu,X.-Y. and Levy,D.E. (1990) Interferon-α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. *Genes Dev.*, **4**, 1753–1765.

Kishimoto,T., Taga,T. and Akira,S. (1994) Cytokine signal transduction. *Cell*, **76**, 253–262.

Kontanides,H. and Reich,N.C. (1993) Requirement of tyrosine phosphorylation for rapid activation of a DNA binding factor by IL-4. *Science*, **262**, 1265–1267.

Larner,A.C., David,M., Feldman,G.M., Igarashi,K., Hackett,R.H., Webb,D.A., Sweitzer,S.M., Petricoin,E.F. and Finbloom,D.S. (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science*, **261**, 1730–1733.

Larose,L., Gish,G. and Pawson,T. (1995) Construction of an SH2 domain binding site with mixed specificity. *J. Biol. Chem.*, **270**, 3858–3862.

Lehmann,J., Seegert,D., Strehlow,I., Schindler,C., Lohmann-Matthes,M.L. and Decker,T. (1994) Interleukin-10 induced factors belonging to the p91 family of proteins bind to interferon-gamma responsive promoter elements. *J. Immunol.*, **153**, 165–172.

Leung,S., Qureshi,S.A., Kerr,I.M., Darnell,J.E. and Stark,G.R. (1995) Role of STAT2 in the alpha interferon signaling pathway. *Mol. Cell. Biol.*, **15**, 1312–1317.

Levy,D.E., Kessler,D.S., Pine,R.I. and Darnell,J.E. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-α stimulated transcription, reconstituted *in vitro*. *Genes Dev.*, **3**, 1362–1372.

Lütticken,C. *et al.* (1994) Association of transcription factor APRF and protein kinase Jak 1 with the interleukin-6 signal transducer gp 130. *Science*, **263**, 89–92.

Mayer,B.J., Jackson,P.K. and Baltimore,R.A.V.E. (1992) Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding *in vitro* and transforming activity *in vivo*. *Mol. Cell. Biol.*, **12**, 609–618.

Migone,T.-S., Lin,J.-X., Cereseto,A., Mulloy,J.C., O'Shea,J.J., Franchini,G. and Leonard,W.J. (1995) Constitutively activated JAK-STAT pathway in T cells transformed with HTLV-1. *Science*, **269**, 79–81.

Mui,A., Wakao,H., O'Farrell,A.M., Harada,N. and Miyajima,A. (1995) Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two Stat5 homologues. *EMBO J.*, **14**, 1166–1175.

Pernis,A., Gupta,S., Yopp,J., Garfein,E., Kashleva,H., Schindler,C. and Rothman,P. (1994) γ chain-associated cytokine receptors signal through distinct transducing factors. *J. Biol. Chem.*, **270**, 14517–14522.

Pine,R., Canova,A. and Schindler,C. (1994) Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN-α and IFN-γ, and is likely to autoregulate the p91 gene. *EMBO J.*, **13**, 158–167.

Quelle,F.W., Thierfelder,W., Witthuhn,B.A., Tang,B., Cohen,S. and Ihle,J.N. (1995) Phosphorylation and activation of the DNA binding activity of purified Stat1 and the Janus protein tyrosine kinases and the Epidermal Growth Factor receptor. *J. Biol. Chem.*, **270**, 20775–20780.

Ralph,S.J., Wines,B.D., Payne,M.J., Grubb,D., Hatzinisiriou,I., Linnane,A.W. and Devenish,R.J. (1995) Resistance of melanoma cell lines to interferons with reduction of IFN-induced tyrosine phosphorylation. *J. Immunol.*, **154**, 2248–2256.

Rothman,P. *et al.* (1994) Cytokines and growth factors signal through tyrosine phosphorylation of a family of related transcription factors. *Immunity*, **1**, 457–468.

Schindler,C. and Darnell,J.E. (1995) Transcriptional responses to peptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.*, **64**, 621–651.

Schindler,C., Fu,X.-Y., Improta,T., Aebersold,R. and Darnell,J.E. (1992a) Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proc. Natl Acad. Sci. USA*, **89**, 7836–7839.

Schindler,C., Shuai,K., Prezioso,V. and Darnell,J.E. (1992b) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science*, **257**, 809–813.

Schindler,C., Kashleva,H., Pernis,A., Pine,R. and Rothman,P. (1994) STF-IL4: a novel IL-4 induced signal transducing factor. *EMBO J.*, **13**, 1350–1356.

Shuai,K., Schindler,C., Prezioso,V. and Darnell,J.E. (1992) Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91-kDa DNA binding protein. *Science*, **258**, 1808–1812.

Shuai,K., Horvarth,C.M., Tsai-Huang,L.H., Quereshi,S.A., Cowburn,D. and Darnell,J.E. (1994) Interferon activation of the transcription factor stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell*, **76**, 821–828.

Silvennoinen,O., Schindler,C., Schlessinger,J. and Levy,D.E. (1993) Ras-independent signal transduction in response to growth factors and cytokines by tyrosine phosphorylation of a common transcription factor. *Science*, **261**, 1737–1739.

Sliva,D., Wood,T.J., Schindler,C., Lobie,P.E. and Norstedt,G. (1994) Growth hormone specifically regulates serine protease inhibitor gene transcription via γ-activated sequence-like DNA. *J. Biol. Chem.*, **269**, 26208–26214.

Songyang,Z. *et al.* (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767–778.

Songyang,Z. *et al.* (1995) Catalytic specificity of protein tyrosine kinases is critical for selective signaling. *Nature*, **373**, 536–539.

Stahl,N., Farruggella,T.J., Boulton,T.G., Zhong,Z., Darnell,J.E. and Yancopoulos,G.D. (1995) Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science*, **267**, 1349–1352.

Velazquez,L., Mogensen,K.E., Barbieri,G., Fellous,M., Uze,G. and Pellegrini,S. (1995) Distinct domains of the protein tyrosine kinase tyk2 required for binding of interferon-α/β and signal transduction. *J. Biol. Chem.*, **270**, 3327–3334.

Wakao,H., Gouilleux,F. and Groner,B. (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.*, **13**, 2182–2191.

Waksman,G. *et al.* (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature*, **358**, 646–653.

Yan,H., Krishnan,K., Greenlund,A.C., Gupta,S., Lim,J.T.E., Schreiber, R.D., Schindler,C. and Krolewski,J. (1996) Phosphorylated interferon-α receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. *EMBO J.*, **15**, 1064–1074.

Zhang,X., Blenis,J., Li,H.-C., Schindler,C. and Chen-Kiang,S. (1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science*, **267**, 1990–1994.

Zhao,Y., Wagner,F., Frank,S.J. and Kraft,A.S. (1995) The amino-terminal portion of the Jak2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor βc chain. *J. Biol. Chem.*, **270**, 13814–13818.

Zhong,Z., Wen,Z. and Darnell,J.E. (1994) Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc. Natl Acad. Sci. USA*, **91**, 4806–4810.

Received on May 30, 1995; revised on October 3, 1995