Identification of Amino Acid Residues Critical for the Src-homology 2 Domain-dependent Docking of Stat2 to the Interferon α Receptor*

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The interaction between Src-homology 2 domains (SH2) domains and phosphorylated tyrosine residues serves a critical role in intracellular signaling. In addition to the phosphotyrosine, adjacent residues are critical mediators of the specificity of this interaction. Upon treatment of cells with interferon α (IFNα), the IFNRα1 subunit of the IFNα receptor becomes tyrosine phosphorylated at position 466. The region surrounding phosphorylated tyrosine 466 subsequently acts as a docking site for the SH2 domain of Stat2, facilitating phosphorylation of the latter and, thus, the transduction of the IFNα signal. In this report site-specific mutagenesis was employed to analyze the nature of the interaction between the SH2 domain of Stat2 and the region surrounding tyrosine 466 on IFNRα1. Mutation of the valine at the +1 position carboxyl-terminal to tyrosine 466 or of the serine at the +5 position inhibits the association of Stat2 with phosphorylated IFNRα1. Moreover, receptors mutated at either of these two positions act in a dominant manner to decrease IFNα signaling, as assayed by both Stat2 phosphorylation and expression of an IFNα-responsive reporter. The demonstration that these two residues are critical in mediating the interaction between Stat2 and IFNRα1 suggests that STAT proteins might utilize a structurally distinct subset of SH2 domains to mediate signal transduction from the cell surface to the nucleus.

Interferon α (IFNα) is a member of the cytokine superfamily of effector molecules. First identified as an anti-viral agent, IFNα has also been shown to affect cell growth by inducing the transcription of growth inhibitory genes (for a review, see Ref. 1), following interaction with widely expressed cell surface receptors. Two subunits of the IFNα receptor (designated IFNRα1 and IFNRα2) have been identified (2–5), both encoded by genes localized within a 400-kilobase region on chromosome 21 (6). As with other cytokine receptors, both subunits are rapidly phosphorylated on tyrosine following binding of the cognate ligand (7–9).

Ligand binding activates transcription via a signaling pathway mediated by members of the Janus kinase and signal transducers and activators of transcription (JAK and STAT) families of proteins. The JAK family of tyrosine kinases includes four mammalian members. Genetic evidence has demonstrated that two of these, Tyk2 and Jak1, are involved in IFNα signal transduction (10, 11). Each of these JAK kinases has been shown to constitutively bind to a subunit of the IFNα receptor; Tyk2 associates with IFNRα1 (12–14) and Jak1 with IFNRα2 (15). In vitro, all of the JAK kinases can phosphorylate IFNRα1, predominantly on tyrosine 466 (Tyr-466) (13, 16). Homodimerization of a chimeric protein composed of the CD4 extracellular domain fused to the IFNRα1 intracellular domain resulted in its tyrosine phosphorylation. This phosphorylation was, again, found to occur primarily on Tyr-466 and to be dependent on the association of IFNRα1 with Tyk2 (17). The pattern of phosphorylation of IFNRα1 under physiologic conditions, though, has not been determined.

The STAT proteins are a family of latent cytoplasmic transcription factors employed in signaling pathways activated by various cytokines and growth factors (for a review, see Ref. 18). STAT proteins share a common domain structure; each has a DNA-binding domain, a transactivation domain, a Src-homology 3 domain, a Src-homology 2 (SH2) domain for binding phosphotyrosine, and a single tyrosine phosphorylation site near the C terminus. Complementation of mutant cell lines nonresponsive to IFNα revealed that two STAT family members, Stat1 and Stat2, are involved in the IFNα signal transduction pathway (19, 20). After IFNα stimulation, Stat2 docks to IFNRα1 via an interaction between the Stat2 SH2 domain and the phosphorylated Tyr-466 (TyrP-466) of IFNRα1 (16, 17). The critical role of TyrP-466 and the Stat2 SH2 domain in this docking interaction has been demonstrated by in vitro binding studies (16). In addition, phosphopeptides corresponding to the region surrounding Tyr-466 and dominant-negative IFNRα1 constructs in which Tyr-466 has been mutated to phenylalanine inhibit Stat2 phosphorylation in vivo (16). Phosphotyrosine dependent recruitment by IFNRα1 positions Stat2 to become tyrosine phosphorylated, presumably by one of the associated JAK kinases. Phosphorylated Stat2, in turn, provides a docking site for the SH2 domain of Stat1, positioning it for tyrosine phosphorylation (16, 19). The two STAT molecules heterodimerize via SH2-phosphotyrosine interactions, translocate to the nucleus, and effect transcription of genes under control of interferon-stimulated gene response elements (ISREs) (21–23).

SH2 domains were first identified as conserved noncatalytic domains in the Src and Fps proteins (24). These domains were subsequently found in many proteins and shown to mediate interactions between cytoplasmic proteins by binding to phosphorylated tyrosine (25). Site-specific mutations in the residues surrounding tyrosines that function as SH2 domain-docking...
sites (summarized in Ref. 26) and subsequent studies that identified ligands for recombinant SH2 domains from degenerate phosphopeptide libraries indicated that the residue immediately C-terminal and the residue three amino acids C-terminal to the phosphorylated tyrosine (the +1 and +3 positions with respect to the tyrosine), were of critical importance in binding and conferreance of specificity for many SH2 domains (27, 28). In this report, we examine the role of the residues C-terminal to Tyr-466 of IFNaR1 in the docking of Stat2 and subsequent signaling events and demonstrate that the amino acids at positions +1 and +5 relative to Tyr-466 are particularly critical for these molecular events.

EXPERIMENTAL PROCEDURES

DNA Constructs—All of the constructs were expressed from the vector pM72T (29). Plasmid constructs encoding the wild type full-length IFNaR1, the Y466F mutant of full-length IFNaR1, and the wild type CD4-IFNaR1 chimera have been previously described (16, 17). A series of five mutant constructs, which convert residues 467 through 471 to alanine were generated by a single step polymerase chain reaction approach, using a 5' primer containing the appropriate mutation and a wild type 3' primer. Mutant polymerase chain reaction fragments spanning positions 1462–1617 of the IFNaR1 cDNA (5) were first cloned into the pGEM-T vector (Promega) and sequenced to ensure fidelity. NsiI-MfeI restriction fragments, containing the various mutations, were transferred into versions of the receptors cloned in Bluescript (Stratagene). Subsequently, PstI or EcoRI fragments containing the entire mutant full-length or chimeric receptor, respectively, were transferred into the pMT2T expression vector. The β-galactosidase expression plasmid (pCH110) used to control for transfection efficiency, and the ISRE-luciferase reporter plasmid (pZ-ISRE-luc; from R. Pine, Public Health Research Institute, New York, NY), which contains four tandem copies of the ISRE, have been previously described (30, 31).

Cell Culture and Transfection—The human embryonic kidney 293T cell line and the human osteogenic sarcoma U2OS cell line were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA). Transfections were performed using calcium phosphate as described previously (16).

Antibodies, Immunoprecipitation, and Immunoblotting—The monoclonal anti-CD4 antibody used for cross-linking (Leu1) was obtained from Becton-Dickinson Immunocytometry. Rabbit polyclonal anti-CD4 antiserum were used for immunoblotting (32) were from R.W. Sweet (Smith Kline Beecham Pharmaceuticals). The rabbit polyclonal anti-Stat2 antiserum, used for immunoprecipitation and immunoblotting (22), was from C. Schindler (Columbia University, New York, NY). The anti-Stat2 monoclonal antibody used in the co-immunoprecipitation experiments was obtained from Transduction Laboratories (Lexington, KY). The polyclonal anti-Tyk2 antiserum used for immunoprecipitation and immunoblotting have been previously described (8). The monoclonal anti-anti-phosphotyrosine antibody, 4G10, was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Preparation of lysates, immunoprecipitation, electrophoresis, and immunoblotting were performed as described previously (16, 17).

Fluorescence-activated Cell Sorting—Transfected cells (106) were stained with isotype-matched phycoerythrin-conjugated anti-CD4 and anti-IgG2a murine monoclonal antibodies (CalTag, S. San Francisco, CA) and analyzed as described (14).

Luciferase Assay—For each individual assay, duplicate subconfluent 10-cm dishes of U2OS cells were transfected with 5 µg of the β-galactosidase expression plasmid, 5 µg of the ISRE-luciferase plasmid, and 10 µg of the expression vector. 30 h after transfection, one dish was treated with 3000 units/ml of IFNα2 from M. Brunda; Hoffman-La Roche, Nutley, NJ) for 18 h at 37 °C, and the other dish was left untreated. Cells were then washed twice with cold phosphate-buffered saline, lysed, and rapidly frozen on dry ice. Lysates were thawed, and cellular debris was pelleted by centrifugation at 10,000 × g for 5 min at room temperature. Luciferase activity was measured as per the manufacturer's protocol (Promega) and β-galactosidase activity was measured as described (30).

Statistical Analysis—Data from the luciferase assays was analyzed using an unpaired t test to compare wild type transfecteds with each of the mutants.

RESULTS

To study the contribution of various amino acid residues in the SH2-mediated docking of Stat2 to the IFNaR1 receptor, we took advantage of a chimeric receptor system that reconstitutes the initial events in IFNs signaling, including kinase activation, receptor phosphorylation, and STAT docking (17). More distal events, such as Stat2 tyrosine phosphorylation, are not observed in this chimeric system, presumably because additional receptor subunit(s) (33) are required. Cells transfected with chimeric molecules composed of the CD4 extracellular domain fused to the intracellular domain of IFNaR1 (CD4-IFNaR1) are tyrosine phosphorylated, predominantly at Tyr-466 of IFNaR1, following cross-linking with an anti-CD4 antibody (17). Stat2 is recruited to the phosphorylated chimera, as evidenced by the co-immunoprecipitation of the chimeric receptor by anti-Stat2 antibodies (17). Co-immunoprecipitation was demonstrated to be specific for Stat2 and dependent on the phosphorylation of Tyr-466. In agreement with the in vitro and in vivo data cited above (16), this association supports the idea that Stat2 is recruited to phosphorylated Tyr-466 on IFNaR1. Furthermore, because more distal signaling events do not occur in the chimeric system, the Stat2-IFNaR1 association is unusually stable and can be much more readily detected than the equivalent interaction occurring under physiologic conditions.

We therefore reasoned that any mutation altering a site required for SH2-receptor interaction will inhibit the co-immunoprecipitation of Stat2 and the CD4-IFNaR1 chimera. Five mutant CD4-IFNaR1 chimeric molecules were constructed, substituting alanine for each of the five amino acids immediately C-terminal to Tyr-466 (amino acids 467 through 471, corresponding to the sequence VFFPS). To ensure that the mutations do not effect induced tyrosine phosphorylation, we first performed cross-linking experiments with the mutants and measured chimeric receptor phosphorylation. As the top panel of Fig. 1 shows, anti-CD4 treatment of 293T cells transfected with either the wild type chimera or any of the five mutant constructs results in a strong induced tyrosine phosphorylation of the chimeric receptor. Stripping the anti-phosphotyrosine immunoblot and reprobing with a polyclonal anti-CD4 antibody (Fig. 1, bottom panel) showed that each of the chimeric constructs was expressed, indicating that these single amino acid substitutions do not dramatically alter protein stability. In addition, fluorescence-activated cell sorting analysis with an anti-CD4 antibody showed that the each of the various chimeric receptors was expressed on the cell surface in approximately equivalent levels (data not shown). It is important to note that, although we have observed differences in the level of expression of the various chimeric constructs, the extent of tyrosine phosphorylation is similar in all cases. IFNaR1-Stat2 docking is completely dependent on tyrosine phosphorylation of the chimeric receptor. Thus, this is the most relevant control criteria for comparing the constructs in the recruitment assay described below.

Having demonstrated that mutation of residues adjacent to Tyr-466 does not affect IFNaR1 phosphorylation, we sought to determine which, if any, of these residues influence the association of the Stat2 SH2 domain with the phosphotyrosine on IFNaR1. To test this, wild type and mutant CD4-IFNaR1 chimeras were transfected into 293T cells and cross-linked. Lysates were immunoprecipitated with an anti-Stat2 monoclonal antibody and immunoblotted with an anti-CD4 antisera. As shown in Fig. 2, co-immunoprecipitation and therefore association of CD4-IFNaR1 with Stat2 was detected at similar levels in cells transfected with the wild type construct (lane 1), as well as constructs expressing the mutants F468A (lane 5), F469A (lane 7), and P470A (lane 9). On the other hand, receptors encoding two mutant constructs, V467A (lane 3) and S471A (lane 11), were co-immunoprecipitated significantly less efficiently than the wild type. Thus, three residues (+2, +3, and...
14 with respect to Tyr-466) have little effect on the binding of the Stat2 SH2 domain to IFNaR1, whereas two others (11 and 15) appear to be important for this association. To demonstrate the importance of the 11 and 15 residues in a more physiologic setting, we employed an IFNα-driven reporter gene system. As noted in the Introduction, docking of Stat2 to the phosphorylated IFNaR1 is believed to position Stat2 so that it may be tyrosine phosphorylated by one of the JAK kinases. Phosphorylated STAT molecules dimerize, translocate to the nucleus and stimulate transcription of genes located downstream of an ISRE. Overexpression of a mutant receptor proficient in IFNα binding but deficient in Stat2 docking, we reasoned, would have a dominant inhibitory effect on IFNα signaling.

Adenovirus-transformed 293T cells cannot be employed in reporter gene assays because the E1A gene product binds p300/cAMP response element-binding protein and prevents Stat2-mediated transactivation (34). Therefore, we transfected U2OS cells with a plasmid containing the firefly luciferase gene downstream of an ISRE. Overexpression of a mutant receptor proficient in IFNα binding but deficient in Stat2 docking, we reasoned, would have a dominant inhibitory effect on IFNα signaling.

FIG. 1. Wild type and mutant CD4-IFNaR1 chimeras are tyrosine phosphorylated after cross-linking with anti-CD4. Cells (293T) were transfected with either the wild type CD4-IFNaR1 chimera (WT) or one of the five constructs containing the indicated mutations. Two days later, transfectants were split and one-half was left untreated (odd numbered lanes), whereas the other half was treated with an anti-CD4 antibody (Leu3a) to cross-link the chimeric receptors (even numbered lanes). Lysates were immunoprecipitated with an anti-CD4 antibody (OKT4) and immunoblotted with an anti-phosphotyrosine antibody (upper panel). The position of phosphorylated CD4-IFNaR1 is indicated. Slower migrating bands in lanes 3 and 5 represent an artifactual cross-reactive species that has been observed previously (17). Blots were stripped and reprobed with an anti-CD4 polyclonal antibody (lower panel). The position of the receptor protein is indicated.

FIG. 2. Mutations in amino acids near the major site of tyrosine phosphorylation of IFNaR1 affect co-immunoprecipitation with Stat2. Cells (293T) were transfected with either the wild type CD4-IFNaR1 chimera (WT) or one of the five constructs containing the indicated mutations. Two days later, transfectants were split and one-half was treated with an anti-CD4 antibody (Leu3a) to cross-link the chimeric receptors (odd numbered lanes), whereas the other half was left untreated (even numbered lanes). Lysates were immunoprecipitated with an anti-Stat2 monoclonal antibody, electrophoresed on a nonreducing gel, and immunoblotted with an anti-CD4 antibody to assay co-immunoprecipitation of the chimeric molecules with Stat2 (upper panel). The position of the chimera is indicated. The filter was stripped and reprobed with a polyclonal anti-Stat2 antibody to control for the efficiency of immunoprecipitation (lower panel). The position of the Stat2 protein is indicated. The intense band seen in some lanes of the lower panel, which migrates more slowly than Stat2, is nonreduced immunoglobulin.

Key Residues in a Stat2 Docking Site
Key Residues in a Stat2 Docking Site

Fig. 3. Overexpression of mutant IFNaR1 proteins inhibit IFNo-mediated transcriptional activation. Cells (U2OS) were co-transfected with either wild type IFNaR1 (WT) or IFNaR1 constructs containing the indicated mutations, a plasmid containing the firefly luciferase gene downstream of ISRE control elements and a plasmid encoding a constitutively expressed β-galactosidase gene. 30 h post-transfection, one-half of the transfected culture was treated with 3000 units/ml IFNa for 18 h at 37 °C, whereas the other half was left untreated. Lysates were prepared and analyzed for luciferase and β-galactosidase activities. Plots represent fold increase in IFNα-mediated transcriptional activation relative to untreated cells, normalized for transfection. Wild type and mutant IFNaR1 constructs were overexpressed in 293T cells. These cells were used because they are IFNo responsive and can be transfected at the very high levels required to permit the proteins produced by the transfected genes to displace endogenous molecules involved in signaling interactions (17). Tyrosine phosphorylation of signaling components was assayed by immunoblotting anti-Tyk2 and anti-Stat2 immunoprecipitates with an anti-phosphotyrosine monoclonal antibody (4G10). In cells transfected with either wild type IFNaR1 (Fig. 4A, lane 1) or any of the five IFNaR1 constructs mutated near Tyr-466 (Fig. 4A, lanes 3, 5, 7, 9, and 11), IFNo treatment induces the tyrosine phosphorylation of Tyk2. On the other hand, when Stat2 phosphorylation was assayed, differences were observed between the various mutants. Stat2 phosphorylation in cells transfected with the mutants F468A, F469A, and P470A (Fig. 4B, lanes 5, 7, and 9, respectively) occurs at levels similar to that of wild type IFNaR1 (Fig. 4B, lane 1). On the other hand, cells transfected with mutants V467A and S471A (Fig. 4B, lanes 3 and 11, respectively) showed a decreased induction of Stat2 tyrosine phosphorylation, relative to the wild type. Tyrosine phosphorylation of the Stat1 protein, which co-immunoprecipitates with Stat2, is also diminished.

As shown previously (16), overexpression of the Y466F mutant of IFNaR1 has a stronger effect on IFNo induced Stat2 and Stat1 tyrosine phosphorylation (Fig. 4C). Thus, consistent with the idea that SH2-dependent docking on IFNaR1 positions Stat2 for tyrosine phosphorylation, mutations at the +1 and +5 positions show a dominant inhibitory effect on Stat2 activation, independent of Tyk2 activation. Furthermore, as in Fig. 3, IFNaR1 constructs containing mutations in the carboxyl-terminal flanking amino acids produce a more modest dominant negative effect compared with that observed with a construct in which the critical tyrosine has been mutated to phenylalanine.

**DISCUSSION**

We have previously demonstrated that following IFNo treatment, Stat2 is specifically recruited to Tyr(P)-466 on the IFNaR1 subunit of the receptor (16, 17). SH2-mediated recruitment of STATs to phosphorylated cytokine receptors is believed to be the mechanism by which STAT specificity is determined (35). To confirm that the region surrounding Tyr-466 is required for Stat2 recruitment, and to begin to characterize the residues that confer specificity for Stat2 binding, we systematically mutated the five amino acids C-terminal to Tyr-466 on the IFNaR1 receptor subunit. We employed two experimental approaches previously used to characterize the IFNo-signaling
pathway: (i) inducible activation of a chimeric receptor by anti-CD4 antibodies (17), and (ii) overexpression of dominant inhibitory versions of IFNaR1 (16). Our results indicate that residues at the 11 and 15 positions C-terminal to Tyr-466 are critically important in mediating the binding of Stat2 to Tyr(P)-466. Specifically, mutation of either of these residues to alanine did not effect the anti-CD4 induced tyrosine phosphorylation of the chimeric receptor (Fig. 1) but did drastically disrupt the ability of the receptor to co-immunoprecipitate Stat2 (Fig. 2).

Second, full-length receptors with alanine at either position function in a dominant inhibitory fashion to suppress both ISRE-dependent reporter gene activity (Fig. 3) and Stat2 tyrosine phosphorylation (Fig. 4B) in response to IFNα. Significantly, overexpression of constructs with these same mutations did not suppress Tyk2 tyrosine phosphorylation (Fig. 4A), consistent with the idea that the effect we have observed is occurring at the level of Stat2 docking to the IFNaR1 subunit and not in earlier steps in the signaling cascade, such as kinase activation.

Stark, Kerr, and colleagues (36) have produced mutant cell lines that are deficient in various components of the IFNo signaling pathway. Complementation of these mutant lines proved invaluable in establishing the role of these components under physiologic conditions. Unfortunately, no IFNaR1-deficient mutants have been identified, preventing us from performing similar experiments. In addition, the murine IFNaR1 receptor does not contain an obvious region of homology to the Tyr-466 docking site on the human version, nor has a functionally equivalent site been identified (37) (it is unclear how Stat2 is recruited to the murine receptor). Thus, complementation experiments, which might confirm our results, await the development of human cell lines that are null at the IFNaR1 locus.

Although residues carboxyl-terminal to the tyrosine contribute to the specificity of an SH2-phosphotyrosine interaction, the strongest binding occurs between the SH2 domain and the phosphotyrosine itself, which fits into a deep pocket containing a positively charged arginine residue (38). Thus, Tyr(P)-466 is the main determinant of affinity for Stat2 when it binds the docking site on IFNaR1. As such, mutation of residues carboxyl-terminal to the phosphorylated tyrosine will likely weaken, but not entirely abrogate Stat2 binding. This is consistent with our observation of some residual co-immunoprecipitation for the +1 and +5 mutant constructs in Fig. 2. Other explanations, such as low level binding of Stat2 to the region surrounding Tyr-481 (16) or an incomplete dominant negative effect because of technical limitations might also account, at least in

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**FIG. 4.** Overexpression of mutant IFNaR1 proteins inhibit IFNo-induced Stat2 tyrosine phosphorylation. Cells (293T) were transfected with either wild type IFNaR1 (WT) or the mutant construct indicated. Two days post-transfection, cells were harvested and one-half of each transfected culture was treated with IFNα at 1000 units/ml for 10 min at 37 °C (odd numbered lanes), whereas the other half was left untreated (even numbered lanes). Panel A, lysates were immunoprecipitated with an antisera against Tyk2, and immunoblots were probed with an anti-phosphotyrosine antibody (upper panel). The position of phosphorylated Tyk2 is indicated. The filter was stripped and reprobed with an anti-Tyk2 antibody (lower panel). Panel B, lysates were immunoprecipitated with an antisera against Stat2, and immunoblots were probed with an anti-phosphotyrosine antibody (upper panel). The positions of phosphorylated Stat2 and Stat1 are indicated. The band migrating faster than Stat2 is a nonspecific cross-reactive artifact that has been noted previously (16). The filter was stripped and reprobed with an anti-Stat2 antibody (lower panel). Panel C, a separate experiment was performed in the same manner as panel B, except that the wild type and Y466F mutant IFNaR1 constructs were employed.
part, for the residual co-immunoprecipitation. The secondary role of the carboxyl-terminal residues is also well demonstrated in the reporter gene assay, where the Y466F mutant is clearly a stronger inhibitor than either the +1 or +5 mutants (Fig. 3), as well as the dominant inhibition of Stat2 tyrosine phosphorylation (Fig. 4).

The proline residue at the +4 position may also contribute to the Stat2 SH2 domain binding site on IFNaR1. This is evidenced by the modest, but statistically significant dominant inhibitory effect of an alanine mutation at this position on the ISRE-driven reporter gene assay (Fig. 3). In contrast, two biochemical measures of the interaction, Stat2-IFNaR1 co-immunoprecipitation (Fig. 2) and Stat2 tyrosine phosphorylation (Fig. 4), showed little or no difference for the +4 alanine mutation when compared with the wild type controls. One possible explanation for the difference between the reporter assay and the biochemical assays may lie in the increased sensitivity of reporter gene activation to small alterations in phenotype. Another possibility is that the biochemical measurements are made at a single point in time and do not necessarily reflect the kinetics of signaling, whereas reporter gene activation measures the accumulated activation of the signaling pathway.

It should be noted that our results are based entirely on the use of alanine substitution to identify residues that are critical for the Stat2-IFNaR1 interaction. Although alanine is widely employed as a substitute amino acid because its methyl group side chain is unlikely to participate in most hydrophobic or hydrophilic interactions, we cannot rule out the possibility that mutation of the residues in question to amino acids other than alanine might affect the interaction. It is of interest to note that experiments aimed at identifying a consensus sequence for Stat2 SH2 domain binding partners, using a partially degenerate library of phosphopeptides, were not successful because of technical limitations (28). Thus, additional data that might guide the choice of other substitutions is lacking at present.

Co-crystallization of the Src SH2 domain with a phosphotyrosine containing peptide ligand corresponding to a known high affinity binding site revealed not only a conserved binding pocket for phosphotyrosine but also a set of interactions between the SH2 domain and residues of the peptide carboxyl-terminal to the tyrosine (39). The amino acid immediately carboxyl-terminal to the tyrosine (the +1 residue) was found to make direct contact with the side chains of two amino acids (D3 and βD5) within the βD-strand of the central β-sheet conserved in SH2 domains. SH2 domains with glutamine at the βD3 position preferentially bind peptides with valine, leucine, or isoleucine at the +1 position (27). Stat2 contains a glutamine at the position corresponding to βD3 (40) and binds to a phosphotyrosine motif containing a valine at the +1 position. As shown in the Fig. 2, mutation of this valine to alanine substantially reduces the association between Stat2 and phosphorylated IFNaR1. The Stat2 SH2 domain also interacts with a phosphorylated tyrosine on the Stat1 protein (Tyr-701) when the Stat1-Stat2 heterodimer is formed (21, 41). Interestingly, the +1 position with respect to Tyr-701 of Stat1 is an isoleucine, one of the amino acids predicted to interact with a βD3 glutamine (27). Thus, our finding that Val-467 on IFNaR1 is important for Stat2 SH2 binding fits with one of the predicted structural features of the Stat2 SH2 domain.

X-ray crystallographic data also revealed that the SH2 domains of both Src (38, 39) and Lck (42) fold to create a hydrophobic pocket into which the side chain of the +3 amino acid fits. Other SH2 domains were subsequently found to make direct contacts with the +3 position. However, mutation of the +3 site in IFNaR1 (Phe-469) to alanine does not affect association of the receptor with Stat2 (Fig. 2), nor does it effect downstream signaling (Figs. 3 and 4). In contrast, our data suggest that the serine in the +5 position is a likely site of contact between the Stat2 SH2 domain and IFNaR1. The involvement of a residue at the +5 position in SH2-phosphotyrosine interaction is not without precedent. For example, x-ray crystallographic analysis of the N-terminal SH2 domain of Syp complexed with high affinity peptides revealed strong interaction between the SH2 domain and the +5 residue of the phosphopeptide (43). This SH2 domain, however, also forms a hydrophobic channel that contacts the +1 and +3 residues, in a manner similar to Src. In fact, the side chain of the +3 residue projects deep within the SH2 domain of Syp, providing extensive contacts and stability. Thus, the structure of the SH2 domain of Syp is unlikely to be analogous to that of the N-terminal SH2 domain of Syg.

The SH2 domain of Stat1 was also recently shown to display a distinctive binding specificity. In a manner similar to IFNα, IFNγ induces tyrosine phosphorylation of its receptor at a specific site, tyrosine 440, which is subsequently used as a docking site for the SH2 domain of Stat1 (44). Using changes in surface plasmon resonance as a measure of binding affinities, Greenlund et al. (45) showed that mutation of the +1 and +4 positions to alanine reduced the affinity of the Stat1 SH2 domain for the IFNγ receptor. The effect of mutation of the +5 position, however, was not tested in this system, and thus at this time it is premature to draw conclusions about the similarity of the binding specificities of these SH2 domains. Maren gere et al. (46) classify SH2 domains into two groups - those that select hydrophobic residues at the +1, +2, and +3 positions; and those that select hydrophilic residues at the +1 and +2 positions, and a hydrophobic amino acid at the +3 position. The data for selectivity of both Stat1 and Stat2, though, indicate that they may be structurally distinct and define a new class or classes of SH2 domains.

In most cases, the role of the SH2 domain is to stably localize a protein to the inner surface of the cell membrane via an interaction with phosphotyrosine on either catalytic receptors or adaptor molecules. The Stat2 SH2 domain, on the other hand, must interact sequentially with at least two separate phosphotyrosines. First, it transiently binds Tyr(P)-466 on IFNaR1. Subsequently, it uncouples from the receptor and dimerizes with Stat1, prior to translocating to the nucleus. Thus, functionally, the STAT SH2 domains are distinct from previously characterized SH2 domains. Although additional studies, including crystallographic analyses, will be required to confirm our hypothesis, the data presented in this report suggest that the SH2 domain of Stat2 may be structurally distinct from previously characterized SH2 domains.

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