Mutational Analysis of the STAT6 SH2 Domain*

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The SH2 domain of the STAT family of transcription factors is essential for STAT binding to phosphorylated cytoplasmic domains of activated cytokine receptors. Furthermore, the same domain mediates dimerization of activated STAT monomers, a prerequisite for DNA binding by this family of proteins. To identify amino acid residues within the STAT protein that mediate these various interactions, we have carried out an extensive mutational analysis of the Stat6 SH2 domain. Recombinant proteins carrying C-terminal deletions or double alanine substitutions were expressed in mammalian and insect cells and assayed for DNA binding, transcription activation, tyrosine phosphorylation, and the ability to interact with a tyrosine-phosphorylated peptide derived from the interleukin-4 receptor signaling chain. From these studies, we have identified amino acids that are required for both DNA binding and interleukin-4 receptor interaction, as well as residues that when mutated impair only one of the two functions. Our results suggest that the structural homology between the SH2 domain of Stat6 and that of the distantly related Src protein may be higher than predicted on the basis of primary amino acid sequence comparisons. However, the two types of SH2 domains may differ at their C-terminal ends.

Tyrosine phosphorylation and specific recognition of these phospho-residues by SH2 domain-containing proteins are critical features of many cellular signaling pathways. Hence, this protein-protein interaction domain has been the focus of many studies (1, 2). The best characterized class of SH2 containing proteins is the Src family. Structural analysis of the Src SH2 domain bound to a high affinity phosphopeptide revealed three key features necessary for selective protein recognition (3, 4). First, phosphotyrosine binding is mediated through a mostly polar pocket that contains the conserved GTFLLR motif found in most SH2 domains. Second, a β-sheet structure interacts with the first two amino acids (i + 1 and i + 2) immediately C-terminal to the phosphotyrosine residue. Third, the i + 3 residue is recognized specifically by a second, more hydrophobic pocket (4). Thus, binding selectivity is determined largely by the three amino acids following the phosphotyrosine and specific residues in the interacting SH2 domain (5, 6). In general these features are shared by all SH2 domain-containing proteins for which structural information is available (6–9).

The STAT proteins are the only transcription factors known to contain SH2 domains (10). Thus far, seven STAT proteins have been characterized. Some are activated by multiple cytokines or growth factors, whereas others are only activated in response to a specific stimulus (11–14). Activation of STAT proteins involves cytokine binding to its receptor, which triggers tyrosine phosphorylation of the intracellular receptor domain by an associated Jak kinase (10, 15, 16). The phosphorylated receptor chain provides a docking site for the latent STAT protein, which resides in the cytoplasm. Once recruited to the receptor, the STAT protein is phosphorylated at a single tyrosine residue by Jak kinase (12). Phosphorylated STAT monomers dimerize, translocate to the nucleus, and modulate transcription through STAT-specific DNA sequence elements (11, 17).

Receptor-associated Jak kinases are relatively nonselective in their ability to phosphorylate individual cytokine receptors and/or STAT proteins (18, 19). Furthermore, many STAT proteins, once activated, bind to similar DNA motifs, although not all STAT proteins can activate transcription from the same motif (11, 17, 20–23). Selectivity in gene activation upon stimulation with different cytokines appears to be achieved at the STAT-receptor interaction (11, 18, 24). This interaction is mediated by the STAT-SH2 domain, which also dictates the specificity in STAT:STAT dimer formation (25–27). The selectivity of Stat1 for the IFN-γ receptor can be transferred to Stat2 via the SH2 domain, suggesting that the STAT-SH2 domain is a modular structure as are other SH2 domains (2, 18, 28). Strikingly, STAT-SH2 domains share little sequence similarity with other SH2 domains (Fig. 1). Most of the similarity is restricted to the N-terminal half which contains the conserved GTFLLR motif (4). As with other SH2 domains, mutation of the invariant arginine leads to loss of phosphotyrosine recognition in STATs (5, 21, 29, 30).

Specific peptide recognition is mediated by residues in the C-terminal half of the SH2 domain (4, 6) where little homology exists between the STAT-SH2 and other SH2 domains (Fig. 1). In addition, no structural information is available for any of the STAT-SH2 domains. Given the involvement of this domain in selective protein-protein interactions, we sought to define aspects of this domain that participate in achieving the fidelity of the Jak/STAT signaling pathway.

We are interested primarily in the IL-4-inducible protein, Stat6. To determine the C terminus of the Stat6 SH2 domain, we generated a series of C-terminal deletion mutants. To identify residues critical for Stat6 function, we carried out a systematic mutational analysis of the SH2 domain by changing two amino acids at a time in the context of the full-length protein. Recombinant mutant proteins were tested for DNA binding, tyrosine phosphorylation, and transcription activation. Proteins were also tested for their ability to interact with a tyrosine-phosphorylated peptide derived from the IL-4 receptor. Mutants that were unable to bind DNA but did interact with the receptor-derived peptide could partially inhibit IL-4-induced gene expression when overexpressed in cells that con-
Unbound peptide was removed by washing the resin 4 times with fragment carrying the indicated 3 flag M2 affinity resin (Kodak). BJAB (10^8) cells were resuspended in retained C-terminal flag epitope tags, which allowed purification on anti-DNA fragment that carried the indicated mutation. In each case, the TPU605; aa 641, TPU604; aa 645, TPU603; aa 650, TPU602) were (TPU389) differs from the previously described construct TPU388 (20) show.

The experiment starts at the highly conserved tryptophan residue. Identical amino acids are highlighted. Gaps (−) were introduced to optimize the alignment. Tyrosine 641 (underlined) in Stat6 is required for activation. Stat6 C-terminal deletion points are marked by arrows, and the numbers of the C-terminal residue are shown.

Experimental Procedures

Cell Culture and Transfections—Bjab cells were grown in RPMI 1640(1×) (Hyclone) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM t-glutamine (Life Technologies, Inc.), and 5 × 10⁻⁵ m β-mercaptoethanol. Stably transfected Bjab cell lines were grown in the same media supplemented with 1 mg/ml G418 (Sigma). Bjab cells were stably transfected with the use of electroporation as described by Tewari and Dixit (31). Human embryonic kidney 293 cells and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech) containing 10% fetal calf serum. Transfections in embryonic kidney 293 cells and HepG2 cells were carried out as described (21). Luciferase and β-galactosidase activity was determined 48 h following transfection using the Promega assay systems. The cells were cultured with 5 ng/ml IL-4 6 h before harvesting.

Expression and Purification of Recombinant STAT Proteins with the Use of the Baculovirus Expression System—Expression constructs for full-length Stat6 (TPU272) and Stat6ΔC (TPU285) carrying a stop codon at position 662 have been described previously (21, 22). All C-terminal deletion mutants (aa 602, TPU601; aa 620, TPU600; aa 633, TPU599; aa 636, TPU598; aa 641, TPU597; aa 645, TPU596; aa 650, TPU595) were generated by replacing the Xmal/SacI fragment of TPU285 with DNA fragments that carried stop codons at the appropriate residues. The fragments were generated with the polymerase chain reaction (PCR). Double amino acid substitutions in the Stat6-SH2 domain were generated by substituting the Xmal/SacI fragment of TPU595 with mutated DNA fragments that were prepared by PCR. The integrity of the resulting clones was determined by DNA sequence analysis. All proteins carried nine histidine residues and a pentapeptide substrate (RRASV) for protein kinase A at their C terminus. With the exception of three mutants (WS, FS, and LY), all proteins were expressed in Hi-5 cells and purified using Ni²⁺ affinity chromatography (22).

Expression and Purification of Recombinant STAT Proteins from Mammalian Cells—The expression construct for full-length Stat6 (TPU389) differs from the previously described construct TPU388 (20) in that a flag epitope tag was introduced at the C terminus. All C-terminal deletion constructs (aa 602, TPU608; aa 620, TPU607; aa 636, TPU605; aa 641, TPU604; aa 645, TPU603; aa 650, TPU602) were generated by replacing the BglII/SpeI fragment of TPU389 with a DNA fragment carrying the indicated 3 deletion. Double alanine mutants were prepared by replacing the BglII/SacI fragment of TPU389 with a DNA fragment that carried the indicated mutation. In each case, the fragments were generated by PCR, and the integrity of the clones was determined by DNA sequence analysis. Mutants WS, FS, and LY failed to express in insect cells and were consequently purified from stably transfected Bjab cell lines. Proteins expressed in mammalian cells contained C-terminal flag epitope tags, which allowed purification on anti-flag M2 affinity resin (Kodak). Bjab (10⁶) cells were resuspended in lysis buffer (30 ml; 0.2 mM NaCl, 30 mM Heps, pH 7.6, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA), incubated on ice for 10 min, and sonicated and clarified by centrifugation at 10,000 × g for 10 min. The supernatant was incubated with anti-flag M2 (1 ml) resin for 1 h at 4°C. Unbound material was washed from the resin with lysis buffer (50 ml). Bound protein was eluted with flag peptide (400 ng/ml).

Peptide Binding Assays—Biotinylated peptides (2.5 mmol: IL-4R peptide, ASSGEEGYPKPQQDLI, or IFN-γ peptide, GGGGGGYPDKPHVL) were coupled to 25 μl of packed streptavidin-agarose beads (Sigma) in 0.5 ml of binding buffer (0.1 mM NaCl, 30 mM Heps, pH 7.6, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA) for 30 min at 4°C. Unbound peptide was removed by washing the resin 4 times with binding buffer (1 ml). Purified protein (20 μg) was incubated with peptide-coupled streptavidin beads (25 μl in a final volume of 500 μl binding buffer) for 90 min at 4°C. Unbound protein was removed by washing the resin 4 times with binding buffer (1 ml) for 10 min each. Bound protein was eluted with 50 μl of SDS sample buffer, and 5 μl were subjected to Western analysis with antibodies directed against Stat6. An aliquot of the starting material (25 μl) was separated on an SDS-polyacrylamide gel, and proteins were visualized by Coomassie R-250 staining to ensure that the input was equivalent for each binding reaction.

In Vitro Phosphorylation of Purified Stat6 Mutants and DNA Binding Assays—Purified Stat6 was activated in vitro with Jak1 kinase (both Stat6 and Jak1 were expressed and purified from insect cells (13)). Phosphorylation conditions were 10 mU Heps, pH 7.4, 50 mM NaCl, 50 mM MgCl₂, 50 μM ATP, 0.1 mM Na₂VO₄, 0.5 μM of Jak1, and 1 μg of Stat6 in a 50-μl reaction volume. Reactions were incubated at room temperature for 30 min. Typically, 1 μl of the reaction was used for mobility shift assays. Nuclear extract preparations and mobility shift assays have been described previously (21).

Immunprecipitations and Phosphotyrosine Blots—Bjab cells (1.5 × 10⁶) stably expressing wild-type Stat6 or mutant derivatives or 293 cells (2 × 10⁶) transiently transfected with Stat6 C-terminal deletion mutants were either treated or not treated with IL-4 for 15 min. Cells were lysed in immune precipitation buffer (50 mM Heps, pH 7.9, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.5% Nonidet P-40), and recombinant STAT proteins were immune-precipitated with anti-flag M2-coupled beads. The beads were washed 5 times with immune precipitation buffer, and bound proteins were eluted in SDS sample buffer (50 μl). Proteins were subjected to Western blotting with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) and anti-flag M2 antibody (Kodak).

RESULTS

Amino Acids C-terminal to Tyr-641 Are Required for Tyrosine Phosphorylation In Vivo—A sequence alignment of the Stat6 and Src SH2 domains is shown in Fig. 1. The conserved tryptophan residue constitutes the N-terminal border of the Src SH2 domain. The GTFLLR motif, which is involved in phosphotyrosine recognition, represents the most conserved region (28). Some sequence identity exists N-terminal to the GTFLLR motif, whereas very little similarity is observed in the C-terminal half. Thus it is impossible to determine the C-terminal border of the Stat6 SH2 domain on the basis of sequence similarity. We showed previously that a truncated version of Stat6 (amino acids (aa) 1–661) that lacks the C-terminal 186 aa binds with wild-type affinity to a tyrosine-phosphorylated peptide derived from the IL-4 receptor (21). This mutant retains the tyrosine residue (aa 641) critical for phosphorylation and dimerization; hence, the protein is able to bind DNA when activated in cells. In order to define more precisely the C-terminal border of the Stat6 SH2 domain, we generated a series of C-terminal deletion mutants by inserting a flag epitope tag and a stop codon at various positions between aa 602 and 650 (Fig. 1). The proteins were expressed in embryonic kidney 293 cells and assayed for DNA binding upon IL-4 stimulation. The 293 cells lack endogenous Stat6 protein but express all other components of the IL-4 signaling pathway. Therefore, any IL-4-inducible DNA binding activity is due to the recombinant Stat6 protein (21). Fig. 2A shows the DNA
binding activity of nuclear extracts prepared from IL-4-treated 293 cells transiently expressing the truncated Stat6 proteins. Only the wild-type protein and the mutant ending with aa 650 bound DNA. Mutants ending with aa 645, 641, and 636 did not bind DNA, although the proteins were expressed at the same level as the wild-type (Fig. 2B). Proteins ending with aa 620 and 602 were not expressed in mammalian cells, suggesting that these truncated proteins are unstable (data not shown). These results indicate that only the mutant ending at aa 650 is, upon phosphorylation, able to dimerize, translocate to the nucleus, and bind DNA. Mutant proteins (ending with aa 645 and 641) retain the critical tyrosine residue but were unable to bind DNA. Hence, we investigated whether these truncated proteins become tyrosine-phosphorylated upon cytokine stimulation. Proteins were immune-precipitated from IL-4-treated 293 cells and probed with anti-phosphotyrosine antibodies (Fig. 2C). Only the DNA-binding positive-derivative ending with aa 650 was tyrosine-phosphorylated. The absence of phosphorylation with mutants 645, 641, and 635 showed that no non-specific tyrosine phosphorylation occurs in response to IL-4. Furthermore, the data suggest that the 9 aa following the critical tyrosine (aa 641) are required for proper cytokine-induced Stat6 activation.

This lack of phosphorylation observed in mammalian cells might reflect an unproductive interaction between the truncated Stat6 proteins and the Jak kinase when both are bound to the IL-4 receptor. To resolve this issue, we investigated whether these same mutants could bind DNA when activated in vitro. The proteins were expressed in insect cells, purified to homogeneity, and tyrosine-phosphorylated in vitro with recombinant Jak1. Again, a deletion mutant ending at aa 650 was able to bind DNA, whereas Stat6 proteins truncated at 641 and 636 were inactive (Fig. 2D). However, while deletion to aa 645 blocked Stat6 phosphorylation in IL-4-treated 293 cells, this mutant was activated in vitro to bind DNA. Thus, the 9 aa following tyrosine 641 are essential for Stat6 activation in vivo, whereas only four of these residues are required for in vitro activation.

**Delineation of the C-terminal Border of the Stat6 SH2 Domain**—We next assessed the C-terminal boundary of the SH2 domain with respect to peptide binding. We previously identified two tyrosine-containing peptides derived from the IL-4 receptor signaling chain that, when phosphorylated (Y^6^KAFS and Y^9^KPFQ), associate specifically with Stat6 (25). Similarly, a tyrosine-phosphorylated peptide derived from the interferon-γ receptor bound specifically to Stat1 but not Stat6 (22, 32). Thus, the specificity of the STAT-receptor interaction can be accurately mimicked with the use of peptides derived from individual receptor chains. We investigated whether truncated Stat6 proteins purified from insect cells could bind specifically to the IL-4 receptor-derived peptide Y^9^KPFQ (S, Fig. 2E). A phosphorylated peptide derived from the interferon-γ receptor served as a negative control (N, Fig. 2E). Biotinylated peptides were attached to streptavidin beads and incubated with purified Stat6 proteins. After binding the beads were washed, and the eluted proteins were analyzed by Western blot. All C-terminal deletion mutants (with the exception of mutant 602 which did not express) bound selectively to peptides with affinities equal to that of wild-type Stat6 (Fig. 2E). Consistent with the peptide binding results is the observation that the mutants exhibited a dominant negative phenotype when overexpressed in an IL-4-responsive cell line (data not shown). Similar results were obtained for Stat6 mutants that retained receptor/peptide binding activity but lacked the ability to bind DNA (21). From the above results we tentatively designated aa 620 as the most C-terminal aa of the Stat6-SH2 domain.

**Alanine Substitutions: Effect on DNA Binding and Transcription Activation in Vivo**—To identify more precisely amino acid residues critical for function of the Stat6 SH2 domain, we carried out an extensive mutational analysis. We substituted 2 aa at a time with alanines in the context of the full-length protein. Mutagenesis began at the conserved Trp-533 and ended at Pro-620. The mutants were expressed in lymphoid (BJAB) and nonlymphoid cells (293 cells), and their DNA binding properties and tyrosine phosphorylation status were determined.

Fig. 3 shows the DNA binding activity of these mutants in nuclear extracts prepared from transiently transfected 293 cells following IL-4 treatment. Identical results were obtained with stably transfected BJAB cell extracts (data not shown). Many of the double alanine substitutions did not impair DNA binding. Because dimerization is a prerequisite for DNA binding, a positive DNA binding signal indicates that the SH2 domain of these mutants is capable of mediating both receptor binding and dimerization. However, certain substitutions reduced or completely abolished Stat6 DNA binding. Some of these loss-of-function substitutions target residues that are conserved between the SH2 domains of Stat6 and Src. For example, all changes in the conserved GTFLLR motif completely abolished DNA binding, consistent with the observation that this region is required for phosphotyrosine binding. Other
double amino acid changes in conserved residues (WS, LI, and LY) also abolished DNA binding, whereas changes in the conserved LLLN sequence had no effect. Substitutions in certain regions without homology to Src (SK, DS, IT, IA, EN, IQ, PF, IR, RI, and RD) interfered with DNA binding.

To determine the effect of these mutations on transcription, we overexpressed the proteins in 293 cells in the presence of a luciferase reporter carrying four copies of IL-4 response elements derived from the germ line epsilon promoter. Previously we showed that this reporter is not active in 293 cells in the presence or absence of IL-4 since 293 cells do not contain endogenous Stat6 (21) (Fig. 3, A and B). However, the reporter can be activated through overexpression of recombinant Stat6 and in the presence of IL-4 (wt, Fig. 3B). By using this assay system, we analyzed all SH2 mutants for their ability to activate transcription. A comparison between Fig. 3, A and B, shows that all proteins that are able to bind DNA also activate transcription, whereas mutants that do not bind DNA are not transcriptionally active. In general the mutants were slightly less active than the wild-type protein which may reflect differences in the expression levels. For reasons we do not understand, mutant VT was significantly more active than the wild-type protein. The loss-of-function mutants were analyzed further to determine where in the Stat6 activation cycle they cease to function.

Alanine Substitutions: Effect on Tyrosine Phosphorylation in Vivo—Various reasons could account for the lack of DNA binding seen with some of our mutants. One possibility is that these

Fig. 3. A, effect of double amino acid substitutions on DNA binding. Nuclear extracts were prepared from IL-4-treated 293 cells that had been transfected with constructs encoding wild-type (wt) or mutant Stat6 proteins that carry double amino acid substitutions. Letters above each lane refer to the amino acids that were mutated in the individual proteins. Arrows refer to the Stat6-specific DNA complex or a nonspecific (ns) DNA complex. The extract used in the 1st lane was obtained from unstimulated cells transfected with the wild-type construct. SL and IR occur twice. SL# and IR* designate the DNA-binding positive mutants. Amino acids where either one or both of the two residues are identical to Src are highlighted. B, effect of double amino acid substitutions on transcription activation. Wild-type and mutant proteins (letters below the bars refer to amino acids that were substituted in the context of the full-length Stat6 protein) were coexpressed with the IL-4-inducible reporter construct. Luciferase activity was determined 48 h posttransfection in cells that had been stimulated with IL-4 for 6 h. Numbers reflect the mean values and standard deviations of three independent experiments.
mutants are not phosphorylated in the cell following IL-4 treatment, because they either failed to bind the receptor or bound the receptor-Jak complex in an unproductive manner. In contrast, mutants that do get phosphorylated completed the receptor binding and activation steps but failed subsequently, most likely because they are impaired in dimerization or nuclear translocation.

Stably transfected BJAB cells expressing flag-tagged versions of Stat6 proteins were treated with IL-4, and extracts were prepared. Recombinant Stat6 proteins were immune-precipitated with anti-flag antibodies, and immunoblotted with anti-phosphotyrosine antibodies. The extracts were also probed with anti-flag antibodies to determine the amount of STAT protein present. Wild-type Stat6 and two mutants (GG and DG) that were active in DNA binding were included as controls. As expected, strong IL-4-dependent tyrosine phosphorylation was observed for these three proteins (Fig. 4). The DNA binding inactive mutants SK, FL, IR, FS, EN, IQ, PF, IR, and RD (hereafter referred to as group 1b) bound the IL-4 receptor peptide with wild-type affinity and selectivity (Fig. 5). Specifically bound Stat6 proteins were detected by Western blot.

Six out of nine group 1 mutants (group 1a: SK, LR, FS, EN, IQ, and PF) were unable to bind tyrosine-phosphorylated peptides, suggesting that the failure of these mutants to be activated in cells results from an inability to bind the IL-4 receptor. There were, however, three exceptions; mutant proteins FL, IR, and RD (hereafter referred to as group 2) bound the IL-4 receptor peptide with wild-type affinity and selectivity (Fig. 5). Hence, group 1b mutations interfere with cellular activation of the proteins but have no effect on IL-4 receptor binding.

The group 2 mutants have weak or moderate tyrosine phosphorylation. Three of these mutants (WS, IT, and RI) failed to bind peptide, whereas one mutant (LY) showed weak nonspecific peptide binding properties. These mutants are referred to as group 2a. Multiple explanations could account for the phenotype observed with group 2a mutants. One possibility is that the proteins could become phosphorylated at residues other than Tyr-641. Another is that the peptide binding assay may not mimic all aspects of the interaction between Stat6 and the IL-4 receptor. Other members of group 2 (LI, GT, and IA) showed weak but selective association with the IL-4 receptor peptide, and one of the group 2 mutants (DS) bound with wild-type affinity. These four mutants were classified as group 2b. The reduced level of tyrosine phosphorylation seen with LI, GT, and IA is most likely explained by the impaired recruitment of the proteins to the IL-4 receptor. The strong peptide binding signal but weak phosphotyrosine signal of mutant DS which would explain the decrease in tyrosine phosphorylation of group 1 and 2 mutants. In order to determine whether these DNA-binding inactive proteins can interact with the IL-4 receptor, we purified them from insect cells and assayed their ability to bind a tyrosine-phosphorylated peptide derived from the IL-4 receptor signaling chain. The proteins were expressed as C-terminal deletions (ending at aa 650); this modification does not alter the peptide binding properties of Stat6 (see above). Three mutants (WS, FS, and LY) could not be expressed in insect cells and were purified from mammalian cells. Proteins were incubated with immobilized peptides representing either the specific IL-4 receptor sequence (S) or the nonspecific IFN-γ sequence (N) (Fig. 5). Specifically bound Stat6 proteins were visualized by Coomassie staining (Load). Letters above the lanes refer to specific amino acid positions that were mutated in the individual proteins.

**Fig. 4. Tyrosine phosphorylation of wild-type and mutant Stat6 proteins.** Recombinant Stat6 proteins were immune-precipitated from stably transfected BJAB cells with the use of anti-flag antibodies. Proteins were subjected to Western blots with either anti-phosphotyrosine (anti-P-Tyr) or anti-flag (anti-flag) antibodies. Letters above the lanes refer to amino acids that were mutated in the individual protein. Extracts were prepared from IL-4-treated (+) or unstimulated (−) cells.

**Fig. 5. Peptide binding assay.** Wild-type (aa 1–650) and mutant Stat6 proteins were purified from insect cells or mammalian cells (WS, FS, and LY) and assayed in vitro for their ability to bind a specific tyrosine-phosphorylated peptide (S) derived from the IL-4 receptor, and a nonspecific tyrosine-phosphorylated peptide (N) derived from the IFN-γ receptor. Proteins were incubated with immobilized peptides, and bound proteins were precipitated and analyzed by Western blot with a Stat6-specific antibody (Bound). The amount of starting material was visualized by Coomassie staining (Load). Letters above the lanes refer to specific amino acid positions that were mutated in the individual proteins.
suggested that this mutant may be a poor substrate for the receptor-associated Jak kinase. 

Alanine Substitutions: Dominant Negative Effects in Vivo—On the basis of our data, the lack of DNA binding for both group 1 and 2 mutants can be explained either by the lack of tyrosine phosphorylation and/or the inability of the proteins to interact with the receptor. However, group 2b mutants are tyrosine-phosphorylated and interact with receptor peptide but are still unable to bind DNA. Hence, these mutations may affect the DNA binding and/or dimerization properties of the protein.

We asked next whether mutant Stat6 proteins could be activated in vitro to bind DNA. All proteins belonging to groups 1 and 2 were purified from insect cells, tyrosine-phosphorylated with purified Jak1, and assayed for DNA binding (Fig. 6). Wild-type Stat6 and the DNA-binding positive mutants DR and LA served as positive controls for activation and DNA binding. The three mutants WS, FS, and LY were purified from mammalian cells and tested in this assay (data not shown). With the exception of the mutant EN, all members of group 1 were negative for DNA binding when activated in vitro. The fact that EN can be activated in vitro to bind DNA suggests that alanine substitution at these residues selectively incapacitates the receptor binding function of the SH2 domain, while leaving its dimerization function largely intact.

Interestingly, members of group 2 showed both phenotypes. Three members (DS, IT, and IA) could be activated in vitro to bind DNA, whereas others (RI, LI, and GT) could not. The properties of the various groups of Stat6 mutants are summarized in Fig. 7.

Alanine Substitutions: Dominant Negative Effects in Vivo—Previously we showed that a Stat6 derivative lacking the transcription activation domain is able to completely inhibit the function of the endogenous Stat6 protein in vitro. In contrast, Stat6 mutants that bound the receptor peptide but failed to bind DNA functioned as partial dominant negative as they could only interfere at the receptor binding but not at the DNA binding level. Mutants that abolished peptide binding and DNA binding had no effect (21). Five of our Stat6 derivatives (group 1b: FL, IR, and RD; group 2b: LI and GT) carry mutations that allow receptor peptide binding but completely inhibit DNA binding. Group 1b proteins bind the IL-4 receptor derived peptide very strongly, whereas group 2b proteins were less effective. The question remains whether the in vitro peptide binding assay completely mimics the in vivo interaction between the IL-4 receptor α-chain and Stat6. To address this issue we determined whether these mutants could function as dominant negative proteins in vivo. We overexpressed the mutant proteins in HepG2 cells in the presence of the IL-4 inducible luciferase reporter (21). Fig. 7 shows that all five proteins were able to inhibit the activity of endogenous Stat6 in a dose-dependent manner. Mutant FL was less effective than the other four proteins. Neither one of the five mutants was as active as Stat6ΔC which lacks the transcription activation domain. One other mutant (LR) belonging to group 1a was used as negative control. Hence, the five mutants that bind the receptor-derived peptide but fail to interact with DNA can interfere with the IL-4 signaling pathway in vivo suggesting that the proteins bind the receptor and consequently interfere with the activation of the endogenous Stat6 protein.
cific binding to tyrosine-phosphorylated peptides (22). We have identified residues in the SH2 domain that have differential effects on individual steps of the Stat6 activation pathway. All mutant proteins that bind DNA are also transcriptionally active, whereas no activation was seen with any of the DNA binding defective mutants. Mutants that are unable to bind DNA fell into different groups which are summarized in Fig. 8.

Mutants in group 1a were unable to bind receptor-derived peptides and were not phosphorylated in mammalian cells following IL-4 treatment. Hence, amino acids at these positions are critical for Stat6 function because these mutations blocked all known steps in the activation pathway. Interestingly, one of these mutants (EN) was able to bind DNA after being activated in vitro, implying that the SH2 domain of EN was unable to dock at the IL-4 receptor but could mediate Stat6 dimerization. Given that the Stat6-receptor interaction is the result of a single SH2-phosphotyrosine coupling, and Stat6 dimerization is the result of two SH2-phosphotyrosine couplings, the single coupling receptor interaction may be more sensitive to this mutation than the double coupling dimerization interaction. The fact that phosphorylation in the cell, but not in vitro, is dependent on the association of Stat6 and the IL-4 receptor
may explain why EN can be activated to bind DNA in vitro but not in vivo.

Mutants in group 1b bind the IL-4 receptor-derived peptide in vitro and also function as partial dominant negatives in vivo. Hence, these proteins seem to bind the IL-4 receptor chain and consequently block the recruitment of endogenous Stat6. However, these mutants are not tyrosine-phosphorylated in response to IL-4 stimulation suggesting that these mutations may impair the interaction of Jak kinase with Stat6 after the protein is bound to the IL-4 receptor. Although we have not shown that a specific interaction exists between the Stat6 SH2 domain and one or more of the Jak ligands, studies involving Stat1 and Stat2 suggest that such an association may exist (35).

Mutants belonging to group 2a do not bind the receptor-derived peptide, but to some extent become tyrosine-phosphorylated in cells. Here, our peptide binding assay may not completely mimic the STAT-receptor interaction, suggesting that in vivo the proteins may be recruited to the receptor. Interestingly, one mutant within this group (IT) was able to bind DNA following in vitro activation. Therefore, the phosphorylation observed in vivo is either nonspecific or the mutant is unstable for DNA binding in the cellular environment.

Mutants in group 2b bind the IL-4 receptor-derived peptide and are phosphorylated in response to IL-4 treatment but do not bind DNA after in vitro phosphorylation. These data suggest that group 2b proteins likely have defects in dimerization or they dimerize incorrectly such that a functional DNA binding domain cannot be formed. However, two of these mutants (DS and IA) bound DNA when activated in vitro. This observation suggests that the defect may result from non-specific phosphorylation in vivo, which would yield proteins that are unable to bind DNA. In contrast, the other two mutants (LI and GT) did not bind DNA even after being phosphorylated in vitro. Furthermore, these proteins were able to partially suppress the function of the endogenous Stat6 protein suggesting that the proteins bind the IL-4 receptor in vivo. Based on these observations and the data obtained for the group 1b mutants, we could argue that a slightly different interface may be required for STAT-STAT versus STAT-receptor interactions.

Structural Implications—Structural studies on Src and other SH2 domain-containing proteins have led to a detailed understanding of how SH2 domains selectively recognize different tyrosine-phosphorylated proteins. On the basis of the Src SH2 domain structure, it has been proposed that tyrosine-phosphorylated peptides bind to this domain in a two-pronged manner (4, 6). First, phosphotyrosine binds to one protein pocket that is universal to all SH2 domains and formed by a cluster of residues located in the N-terminal half of the SH2 domain including the GTFLLR motif. Interactions between this pocket and the tyrosine phosphate residue contribute to high affinity binding. Second, the i + 3 residue is bound by a second, more hydrophobic pocket. Formation of this pocket involves residues clustered in the C-terminal half of the SH2 domain, and interactions mediated by this pocket contribute greatly to binding specificity. Additional interactions are mediated by a β-sheet structure, between the two pockets, which makes contacts to the i – 1 and i + 2 positions of the phosphopeptide.

Thus far, no structural information is available for any of the STAT-SH2 domains, and the sequence similarity between the STAT-SH2 domains and those of other signaling molecules is restricted to the phosphotyrosine binding pocket. This lack of sequence similarity may hint at new structural features that are unique to STAT-SH2 domains. By mutating various residues within the Stat6 SH2 domain, we hoped to attain a better understanding of these important issues. We aligned the Stat6 mutant profiles with the Src SH2 domain sequence for which functional residues have been identified based on both structural and mutagenesis studies (Fig. 8D). The loss-of-function that we observed when mutations were introduced into the conserved GTFLLR motif were predictable from the sequence identity. Also, Lys-544 in Stat6 appears to be functionally equivalent to the arginine found in helix 1 of most SH2 domains.

Src residues that mediate selective peptide binding are also indicated in Fig. 8B (3). Although no primary sequence similarity exists, many of our loss-of-function substitutions in Stat6 (ENIQPF) occur in residues at positions that are equivalent to those that mediate sequence-selective Src-peptide interactions (KHKYIR). Alignments between the SH2 domains of STATs and those of other signaling molecules have been suggested and usually require the inclusion of substantial loop structures in order to optimize the alignment (27). Our studies predict that the Stat6 SH2 domain and, possibly, other STAT-SH2 domains share structural features with the Src SH2 domain. Thus, models of known SH2 domain-peptide interactions may be useful for deciphering some features of the Stat6 SH2-peptide interaction.

Based on structural studies, residues in the extreme C-terminal end of the Src SH2 domain make crucial contacts with the i + 3 position of the cognate peptide ligand. Downstream of Tyr-616 in Stat6, none of the extreme C-terminal residues are important for the phosphopeptide binding function of the Stat6 SH2 domain. These observations predict that structural and functional differences between the Stat6 and Src SH2 domains are likely to exist at the extreme C-terminal ends.

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