Immunity, Vol. 2, 677–687, June, 1995, Copyright © 1995 by Cell Press

Stat Recruitment by Tyrosine-Phosphorylated Cytokine Receptors: An Ordered Reversible Affinity-Driven Process

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Summary

Herein, we demonstrate that purified Stat1 binds to its tyrosine-phosphorylated docking site on the IFNγ receptor α chain in a direct, specific, and reversible manner. Using surface plasmon resonance, we determine the affinity (K_D = 137 nM) and specificity of the interaction and define the minimum affinity needed for receptor-mediated Stat1 activation. In addition, we quantitate the relative ability of purified Stat1 to interact with tyrosine-phosphorylated binding sites on other Stat proteins. Finally, we describe experiments that imply that the unidirectional release of activated Stat1 from the IFNγ receptor reflects the preference of free tyrosine-phosphorylated Stat1 monomers to form high avidity reciprocal homodimers rather than reassociating with the receptor binding site. Our results demonstrate that IFNγ-induced Stat1 activation is an ordered and affinity-driven process and we propose that this process may serve as a paradigm for Stat activation by other cytokine receptors.

Introduction

Interferon-γ (IFNγ) is an immunomodulatory cytokine that induces its pleiotropic effects on cells following interaction with a species-specific receptor expressed on cell surfaces (Farrar and Schreiber, 1993). Functionally active IFNγ receptors consist of two species-matched polypeptide chains: a 90 kDa α chain that is necessary and sufficient for ligand binding and necessary but not sufficient for signal transduction, and a 62 kDa β chain that is required for signal transduction (Schreiber and Aguet, 1994).

Work from several laboratories has shown that IFNγ signaling involves the participation of specific members of the Janus kinase–signal transducers and activators of transcription (Jak–Stat) signaling pathway, including the tyrosine kinases Jak-1 and Jak-2 and the latent cytosolic SH2 domain containing transcription factor Stat1 (Darnell et al., 1994; Müller et al., 1993; Watling et al., 1993; Fu, 1992; Shuai et al., 1992). We have recently shown that IFNγ induces the rapid and reversible phosphorylation of the only functionally critical tyrosine residue at position 440 in the intracellular domain of the human IFNγ receptor α chain (Greenlund et al., 1994). Using a cell-free assay system, we showed that a specific IFNγ receptor α chain intracellular domain peptide corresponding to residues 436–444 that contained a phosphotyrosine residue for Y440 inhibited ligand-induced tyrosine phosphorylation and activation of Stat1. Moreover, a biotinylated 436–447 phosphopeptide (but not biotinylated nonphosphorylated peptide nor biotinylated irrelevant phosphopeptides) specifically precipitated Stat1 (and not Stat2) from cellular homogenates. Based on these observations, we proposed that the ligand-dependent phosphorylation of Y440 in the human IFNγ receptor α chain intracellular domain creates a docking site on the receptor for Stat1 and thereby provides the mechanism that couples the activated receptor to its signal transduction system (Greenlund et al., 1994).

The aforementioned studies were conducted in crude cell homogenates. Therefore, it was not possible to determine whether Stat1 interaction with its phosphorylated receptor docking site was direct or mediated via an adapter protein. In the current report, we demonstrate that purified recombinant Stat1 associates directly with the phosphorylated Y440-containing sequence of the IFNγ receptor α chain and quantitate the affinity of the interaction by surface plasmon resonance. Using a hamster monoclonal antibody (MAb) specific for the Stat1 SH2 domain, we show that interaction of Stat1 with the phosphorylated IFNγ receptor α chain occurs through the Stat1 SH2 domain. Finally, using phosphotyrosine containing peptides derived from proteins to which Stat1 binds, a range of affinities is determined that explains and predicts the ordered interactions that occur between Stat1 and its specific ligands.

Results

Generation and Characterization of Purified Recombinant Human Stat1

SDS–PAGE analysis of the purified His-tagged recombinant Stat1 preparations followed by protein staining revealed that the preparations contained a major protein species displaying an M_r of 90 kDa and a minor component (2.5%) of M_r 65 kDa (Figure 1). Western blot analysis performed with polyclonal antisera raised against the amino terminal 65 aa of Stat1 showed that both proteins were Stat1 related. When blots were developed with antisera specific for the Stat1 SH2 domain or the carboxy-terminal 36 aa of Stat1, only the 90 kDa component was recognized. Thus, the lower molecular mass band represented a truncated Stat1 polypeptide lacking the SH2 domain and carboxyl tail.

To document that the purified protein was indeed Stat1, we monitored the ability of the purified Stat1 preparation to bind DNA following exposure to purified Jak-1 kinase and ATP (Figure 1B). Whereas no DNA binding was observed in the untreated purified Stat1 preparation, strong binding activity was expressed in Stat1 preparations following in vitro tyrosine phosphorylation. The gel shift complex formed with purified proteins migrated in a manner

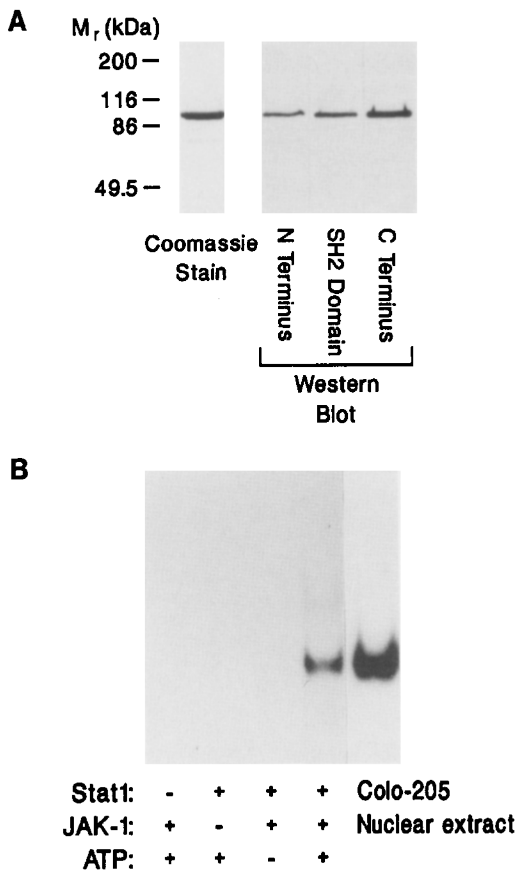

Figure 1. Analysis of Purified Recombinant Human Stat1

(A) SDS–PAGE analysis of purified recombinant human Stat1. Purified Stat1 (2.5 μg) was subjected to SDS–PAGE on a 9% polyacrylamide gel. Subsequent to electrophoresis, the protein was visualized using Coomassie brilliant blue stain. Purified recombinant human Stat1 (100 ng) was also subjected to SDS–PAGE and electrophoretically transferred to nitrocellulose followed by Western blot analysis using rabbit antisera against the amino (N) terminus, SH2 domain, or the carboxy (C) terminus of human Stat1.

(B) Expression of DNA binding activity by purified Stat1 following incubation with purified Jak-1 and ATP. Reaction mixtures containing 1 μg purified Stat1, 1 μg purified Jak-1 tyrosine kinase, or both, were incubated at 23°C for 30 min in kinase reaction buffer in the absence or presence of 200 μM ATP. The formation of a Stat1 complex capable of binding DNA was monitored by subjecting 50% of each reaction mixture to EMSA using a ^{32}P-labeled oligonucleotide probe derived from the GRR of the FcγRI gene. Nuclear extracts (24 μg) derived from IFNγ-treated Colo-205 cells were also subjected to EMSA.

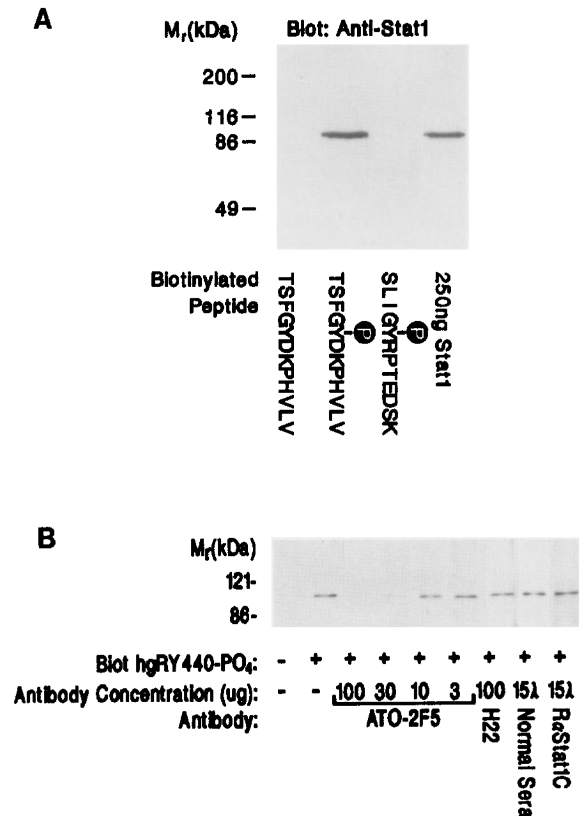

Figure 2. Interaction Between Stat1 and its Tyrosine Phosphorylated Docking Site on the IFNγ Receptor Intracellular Domain Is Direct and Mediated by the Stat1 SH2 Domain

(A) Direct precipitation of purified Stat1 with the IFNγ receptor α chain–derived Y440 containing phosphopeptide. Purified Stat1 (10 nM) was incubated with 100 nM of either biotinylated nonphosphorylated 436–447 peptide (TSFGYDKPHVLV), biotinylated tyrosine-phosphorylated 436–447 peptide (TSFGY-PO_4DKPHVLV), or biotinylated tyrosine-phosphorylated 458–469 peptide (SLIGY-PO_4RPTEDSK). Biotinylated peptides were precipitated with immobilized streptavidin and precipitates examined by SDS–PAGE and Stat1-specific Western blotting. In addition, 250 ng of purified Stat1 was subjected directly to SDS–PAGE and Western blot analysis.

(B) Purified Stat1 (10 nM) was incubated in the absence of MAb or the presence of 100, 30, 10, or 3 μg of the anti-Stat1 SH2 domain–specific MAb ATO-2F5 or 100 μg of irrelevant H22 MAb. Stat1 (10 nM) was also incubated with 15 μl of either normal hamster sera (normal sera) or rabbit antiserum specific for the carboxy-terminal 36 aa of the Stat1 polypeptide (RαStat1C). After a 5 hr incubation, the biotinylated IFNγ receptor-derived 436–447 phosphopeptide was added to the reactions at a final concentration of 33 nM. Peptides were precipitated and precipitates analyzed for the presence of Stat1 as in (A).

that was similar to that of the natural Stat1-containing gel shift complex generated in IFNγ-treated Colo-205 cells. Moreover, polyclonal antiserum specific for the Stat1 carboxy-terminal 36 aa was able to supershift either DNA binding complex (data not shown). Thus, the purified preparation contained a protein that was antigenically and functionally identical with the natural Stat1 molecule.

Purified Stat1 Interacts Directly with the IFNγ Receptor α Chain-Derived 436–447 Phosphopeptide

The ability of highly purified Stat1 to interact with the phosphorylated $Y_{440}DKPH_{444}$ sequence of the IFNγ receptor α chain was determined by coprecipitation analysis using purified biotinylated forms of receptor-derived 12 aa peptides (Figure 2A). Stat1 was not coprecipitated with either biotinylated nonphosphorylated 436–447 peptide (Figure 2A, lane 1) or biotinylated irrelevant phosphotyrosine containing 458–469 peptide (lane 3). In contrast, purified Stat1 was coprecipitated with biotinylated 436–447 peptide containing phosphotyrosine at position 440 (Figure 2A, lane 2). Coprecipitation was highly efficient, since at least 33% of the purified Stat1 was precipitated as determined by comparison of band intensity with a known amount of the

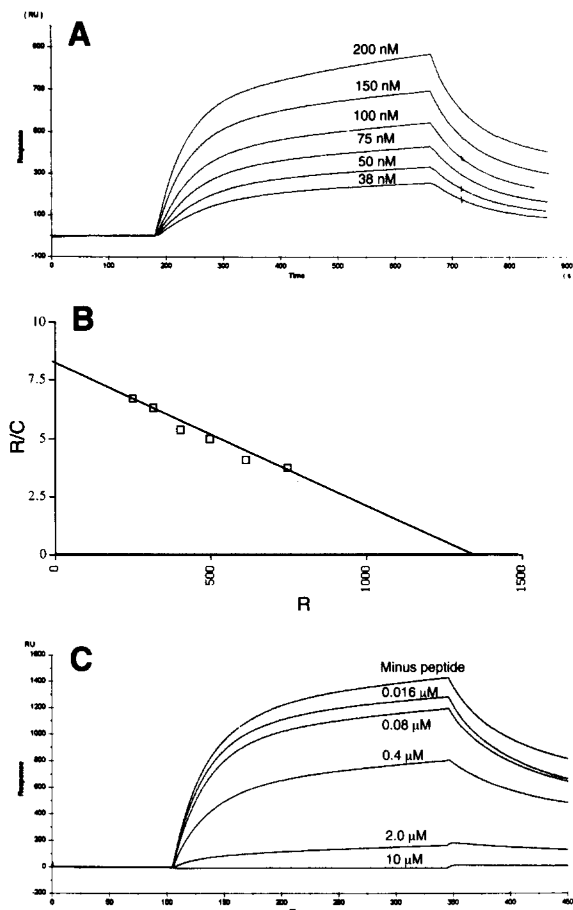

Figure 3. BIAcore Biosensor Analysis of Stat1 Binding to the IFNγ Receptor α Chain–Derived 436–447 Phosphopeptide

(A) Purified Stat1 at concentrations of 38 nM, 50 nM, 75 nM, 100 nM, 150 nM, or 200 nM was injected over a streptavidin sensorchip surface to which the biotinylated IFNγ receptor-derived 436–447 phosphopeptide was immobilized as described in Experimental Procedures. The series of sensograms presented is representative of six independent experiments.

(B) Stat1 steady-state equilibrium binding responses determined from (A) were subjected to Scatchard analysis and the K_D of the Stat1–IFNγ receptor phosphopeptide interaction was determined. The plot represents R/C (steady-state equilibrium response [R_{eq}]/Stat1 concentration [nM]) versus R (steady-state equilibrium response [R_{eq}]).

(C) Purified Stat1 (0.5 μM) was incubated in the absence or presence of the soluble IFNγ receptor-derived 436–446 phosphopeptide at concentrations of 0.016, 0.08, 0.4, 2.0, or 10 μM for 30 min at 10°C. Reaction mixtures were then injected over a sensorchip surface to which the biotinylated IFNγ receptor 436–447 phosphopeptide was immobilized. The initial binding rate of these mixtures was determined and the K_D of the interaction of Stat1 with soluble IFNγ receptor phosphopeptide was calculated as described in Experimental Procedures. The sensograms presented are representative of six independent experiments.

entire purified preparation that was subjected directly to SDS–PAGE and Western blot analysis (Figure 2A, lane 4). Thus, Stat1 interacts directly with its phosphorylated docking site on the IFNγ receptor α chain.

To determine the region of Stat1 involved in phosphopeptide binding, we monitored the ability of antibodies specific for distinct topographical regions of Stat1 to block coprecipitation of the protein with biotinylated phosphorylated IFNγ receptor α chain 436–447 peptide. Coprecipitation of Stat1 was inhibited in a dose-dependent fashion by a hamster MAb ATO-2F5 specific for the Stat1 SH2 domain (Figure 2B). In contrast, coprecipitation of Stat1 was not inhibited by either irrelevant MAb (H22, anti-murine IFNγ), normal hamster serum, polyclonal antiserum specific for the Stat1 carboxyl terminus (RαStat1C) or, in another experiment, polyclonal antiserum specific for the Stat1 amino terminus (data not shown). These results suggest that the interaction between Stat1 and the phosphorylated IFNγ receptor sequence is mediated by the Stat1 SH2 domain. This concept is supported by the observation that a mutant Stat1 protein containing a Lys substitution for the functionally critical R603 residue within the SH2 domain was unable to bind the phosphorylated receptor peptide (data not shown).

BIAcore Biosensor Analysis of Stat1 Binding to the IFNγ Receptor-Derived 436–447 Phosphopeptide

Based on the observation that Stat1 interacted directly with its phosphorylated IFNγ receptor docking site, we determined the affinity of the interaction using surface plasmon resonance. Binding of Stat1 to a sensorchip coated with biotinylated phosphorylated 436–447 peptide was monitored over a Stat1 concentration range of 38–200 nM (Figure 3A). On the basis of six independent determinations, the kinetic on and off rate constants were determined to be $8.6 \pm 0.9 \times 10^4\ s^{-1}M^{-1}$ and $5.7 \pm 1.6 \times 10^{-3}\ s^{-1}$, respectively, leading to a calculated equilibrium dissociation constant of 66 nM. However, because the dissociation portion of the binding reaction was influenced by rebinding of Stat1 to surface-bound phosphopeptide, we empirically determined the K_D by conducting Scatchard analysis of the steady-state equilibrium binding (Figure 3B). Based on six independent experiments, the K_D of the interaction was calculated to be 137 ± 26 nM. To confirm this value, the solution affinity of Stat1–phosphopeptide complexes was determined by incubating Stat1 with concentrations of soluble phosphopeptide ranging from 0.016–10 μM and quantitating the initial binding rate of these mixtures to a phosphopeptide-coated sensorchip surface (Figure 3C). The K_D derived from this analysis was 138 ± 39 nM, which is in good agreement with the value determined by Scatchard analysis.

Stat1 binding was specific. Whereas Stat1 bound to surfaces coated with biotinylated 436–447 phosphopeptide (825 response units [RU]), no binding of Stat1 was observed to surfaces coated with either nonphosphorylated 436–447 peptide (2 RU) or irrelevant 458–469 phosphopeptide (0 RU). In addition, Stat2, a Stat family member that is involved in IFNα signal transduction but not IFNγ signaling did not bind to the IFNγ receptor-derived 436–447 phosphopeptide (2 RU).

Positions +1 and +4 Relative to the Phosphotyrosine Residue Determine the Affinity of Stat1-Phosphopeptide Association

Our previous structure–function work on the IFNγ receptor established that Ala substitution of any one of three IFNγ

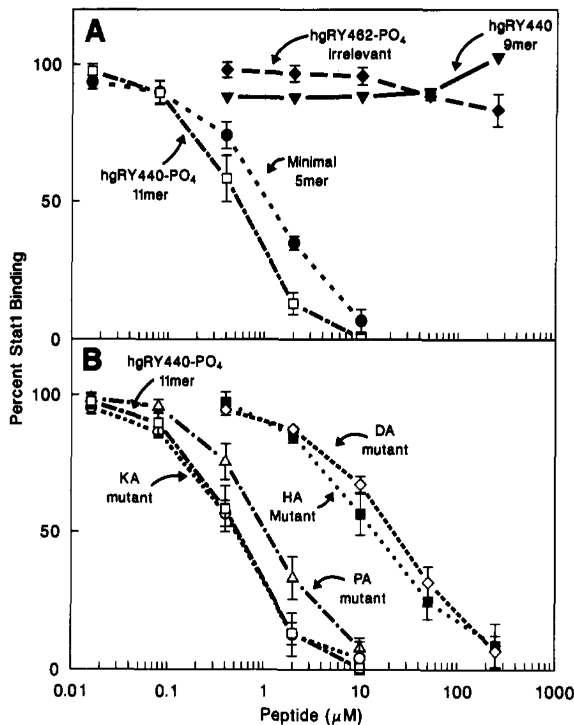

Figure 4. BIAcore Competition Binding Assay to Define the Relative Importance of IFNγ Receptor α Chain Amino Acids in Mediating Stat1 Docking

Purified Stat1 (0.5 μM) was incubated for at least 30 min with IFNγ receptor α chain-derived peptides at concentrations of 0.016, 0.08, 0.4, 2.0, 10, 50, or 250 μM prior to being injected over a sensorchip surface containing immobilized IFNγ receptor α chain-derived biotinylated 436-447 phosphopeptide. The tyrosine-phosphorylated peptides corresponded to residues 436-446 (hgRY440-PO_4, 11-mer, [A and B]), 440-444 (minimal 5-mer, [A]), 458-469 (hgRY462-PO_4, irrelevant, [A]), or 436-446 derivatives (B) containing Ala substitutions for D441 (DA mutant), K442 (KA mutant), P443 (PA mutant), and H444 (HA mutant). The nonphosphorylated peptide contained residues 436-444 (hgRY440, [A]). To avoid bulk refractive index changes due to differences between the injected solution and the elution buffer, the resonance signal 20 s after the end of the injection was monitored. Binding was calculated as the percentage of the total Stat1 binding in the absence of competing peptide. This data represents the mean and standard deviation of at least three independent experiments for each peptide except for the nonphosphorylated IFNγ receptor Y440-containing peptide, which was used as either a 5-mer (data not shown) or a 9-mer (hgRY440, 9-mer) in two separate experiments. The peptide sequences are represented in Table 1.

receptor α chain amino acids (Y440, D441, or H444) produced an IFNγ receptor that was unable to support ligand-induced biologic responses in cells (Farrar et al., 1992). Moreover, we subsequently showed that a receptor-derived phosphopeptide encompassing amino acids 436-447 blocked Stat1 recruitment and activation by ligated IFNγ receptors in cell homogenates, while a mutant phosphopeptide containing Ala substitutions for both D441 and H444 did not (Greenlund et al., 1994). This data unequivocally confirmed the biologic importance of these three amino acids. To define the molecular basis of these observations, we quantitated the ability of nonbiotinylated peptides derived from the Y440 region of wild-type and mutant IFNγ receptors to inhibit binding of purified Stat1 to a sensorchip surface coated with biotinylated phosphorylated IFNγ receptor 436-447 peptide (Figure 4A; Table 1). No significant inhibition was observed with either the nonphosphorylated 436-444 peptide (hgRY440 9-mer) or the phosphorylated 458-469 peptide (hgRY462-PO_4 irrelevant) when used at concentrations up to 250 μM. In contrast, soluble phosphorylated 436-446 peptide efficiently blocked binding of 0.5 μM Stat1 to the 436-447 phosphopeptide-coated surface and displayed an ID_{50} of 0.55 μM (hgRY440-PO_4 11-mer). Comparable inhibition was also observed with a minimal phosphopeptide consisting only of phosphotyrosine$_{440}$-Asp-Lys-Pro-His$_{444}$ (minimal 5-mer, ID_{50} = 1.1 μM). Therefore, this five amino acid sequence constitutes a major portion of the receptor docking site for Stat1.

Table 1. Relative Stat1 Affinities for Sites on Natural and Mutant Tyrosine-Phosphorylated Stat1 Ligands

Peptide	Peptide sequence	ID_{50} (μM)
hgR Y462-PO_4	SLIGY^P RPTEDSK	>250
hgR Y440	TSFGY DKPH--	>250
hgR Y440-PO_4	TSFGY^P DKPHVL	0.55
hgR Y440-PO_4 5mer	----Y^P DKPH--	1.1
hgR DA mutant	TSFGY^P AKPHVL	22
hgR KA mutant	TSFGY^P DAPHVL	0.50
hgR PA mutant	TSFGY^{P}DKAHVL	1.1
hgR HA mutant	TSFGY^P DKPAVL	14
Stat1 Y701-PO_4	KGTGY^P IKTELI	60
Stat1 ID/EH mutant	KGTGY^P DKTHLI	4.3
Stat2 Y690-PO_4	ERRKY^P LKHRLI	7.5
Stat3 Y705-PO_4	SAAPY^P LKTKFI	33
Stat4 Y694-PO_4	GDKGY^P VPSVFI	80
Stat5 Y694-PO_4	AVDGY^P VKPQIK	300
Stat6 Y641-PO_4	DGRGY^P VPATIK	32

ID_{50} (μM) values were obtained for phosphopeptides by inhibiting Stat1 (0.5 μM) binding to immobilized IFNγ receptor α chain 436-447 phosphopeptide with soluble phosphopeptide derivatives using a BIAcore competition assay.

We next examined the extent to which the four amino acids downstream of the phosphorylated Y440 residue contributed to the binding affinity. For this purpose, we used a series of peptides that contained Ala substitutions for amino acids 441-444. This approach was specifically chosen since Ala scanning is an accepted method to measure the contribution of amino acid side chains to protein-protein interactions and we had already established the biological consequences of the substitutions on receptor function (Farrar et al., 1992). Ala substitution of either K442 or P443 did not effect the ability of Stat1 to bind the resulting phosphopeptide (Figure 4B, KA mutant and PA mutant, ID_{50} = 0.50 μM and 1.1 μM, respectively). In contrast, Stat1 bound to phosphopeptides containing Ala substitutions for either D441 (DA mutant) or H444 (HA mutant) with 40- or 25-fold lower affinities, respectively. Thus, a 25- to 40-fold reduction in affinity of Stat1-IFNγ receptor interaction correlates with the loss of IFNγ-dependent signaling observed with Ala-substituted receptor mutants in intact cells (Farrar et al., 1992). These results suggest that the interaction between Stat1 and its docking site on the IFNγ receptor intracellular domain is critically dependent on three receptor contact residues: phosphotyrosine and

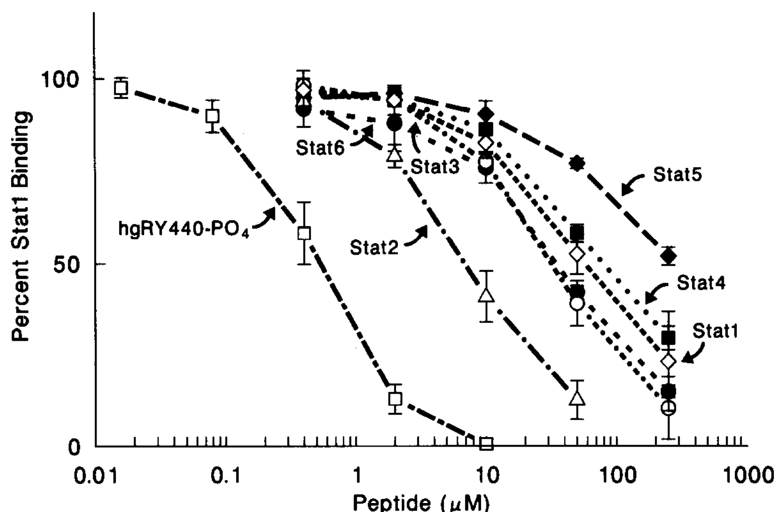

Figure 5. BIAcore Stat1 Competition Binding Assay with Stat-Derived Phosphopeptides

Purified human Stat1 (0.5 μM) was incubated for at least 30 min with peptide concentrations of 0.016, 0.08, 0.4, 2.0, 10, 50, or 250 μM before being injected over a sensorchip surface to which the IFNγ receptor α chain–derived biotinylated 436–447 phosphopeptide was immobilized. The tyrosine-phosphorylated peptides used corresponded to residues 697–707 of human Stat1, 686–696 of human Stat2, 701–711 of human Stat3, 690–700 of murine Stat4, 690–700 of ovine Stat5, 637–647 of human Stat6. The IFNγ receptor α chain–derived tyrosine-phosphorylated peptide corresponded to residues 436–446 (hgRY440-PO_4). To calculate Stat1 binding in the presence of the various phosphopeptide concentrations, the resonance signal 20 s after the end of the injection was monitored. Binding was calculated as the percentage of total Stat1 binding in the absence of competing peptide. This data represents the mean and standard deviation of at least three independent experiments. The sequences of the peptides used in this study are represented in Table 1.

two amino acids at positions +1 and +4 relative to the phosphotyrosine.

Stat1 Binds Phosphopeptides Derived from Stat Proteins

Following interaction with tyrosine-phosphorylated sequences within the intracellular domains of ligated cytokine receptors, Stat proteins become themselves tyrosine phosphorylated and form homo- or heterodimeric Stat complexes (Fu and Zhang, 1993; Greenlund et al., 1994; Shuai et al., 1992, 1993a; Schindler et al., 1992b; Zhong et al., 1994; Akira et al., 1994; Hou et al., 1994; Wakao et al., 1994; Improta et al., 1994). Stat1 dimerization has been the best studied to date, and it is known that phosphorylation of Y701 is the event that leads to Stat1 homodimer formation (Shuai et al., 1993a, 1994). We therefore utilized the BIAcore competition assay to quantitate the interaction of Stat1 with phosphotyrosine containing peptides derived from each of the six recognized Stat family members that included the tyrosine residue within each Stat that is homologous to either Stat1 Y701 (Stat3 and Stat4) or Stat2 Y690 (Stat5 and Stat6) (Figure 5, Table 1). Binding of purified Stat1 to the IFNγ receptor phosphopeptide-coated sensorchip was inhibited by the Stat1 phosphopeptide in a dose-dependent manner. The inhibitory activity of the Stat1 phosphopeptide (ID_{50} = 60 μM) was 110 times lower than that of the IFNγ receptor phosphopeptide (ID_{50} = 0.55 μM). However, if the amino acids in the Stat1 phosphopeptide at positions +1 and +4 relative to the phosphotyrosine (Ile and Glu) were replaced by the corresponding IFNγ receptor amino acids (Asp and His, respectively), the inhibitory activity of the resulting phosphopeptide now approached that of the phosphorylated receptor sequence (ID_{50} = 4.3 μM; Table 1).

Stat1 bound to the Stat2 phosphopeptide with an affinity that was intermediate between the IFNγ receptor and Stat1 peptides (ID_{50} = 7.5 μM). The ability of Stat1 to interact with phosphopeptides derived from Stat3 (ID_{50} = 33 μM), Stat4 (ID_{50} = 80 μM), and Stat6 (ID_{50} = 32 μM) was of similar magnitude to that of the Stat1–Stat1 phosphopeptide interaction. However, Stat1 showed only limited ability to bind to the Stat5 phosphopeptide (ID_{50} = 300 μM). None of the peptides bound to Stat1 when used in nonphosphorylated form (data not shown). These results demonstrate that the latent form of Stat1 interacts preferentially with its docking site on the phosphorylated IFNγ receptor α chain and suggest that the Stat1 SH2 domain may show an ordered preference in its ability to interact with other phosphorylated Stat proteins.

Dimerization of Stat1 Results in a High Avidity Complex

The current concept of Stat protein dimerization is that it occurs as a result of the paired interactions of the phosphotyrosine of one Stat with the SH2 domain of the second (Shuai et al., 1994; Hou et al., 1994). This model predicts that a Stat1 homodimer should be held together with an avidity that is considerably greater than that demonstrated by the individual SH2 domain for phosphotyrosine containing sequences of either Stat1 or the IFNγ receptor.

To evaluate this possibility, we used a cell-free Stat1 electrophoretic mobility shift analysis (EMSA) (Igarashi et al., 1993; Greenlund et al., 1994) in which the IFNγ receptor 436–446 phosphopeptide was added either before or after IFNγ induced Stat1 activation. Addition of phosphopeptide prior to IFNγ treatment inhibited Stat1 DNA binding in a dose-dependent manner (Figure 6). In contrast, substantially higher concentrations of phosphopeptide were required for maximal inhibition of Stat1 DNA binding activity by preformed Stat1 homodimer, i.e., when the IFNγ receptor-derived phosphopeptide was added to cellular homogenates after IFNγ treatment. Nonphosphorylated

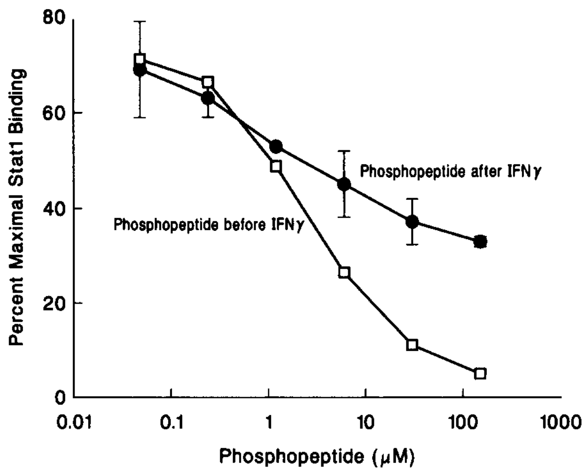

Figure 6. Differential Inhibition of Stat1 Activation and Expression of DNA Binding Activity by the IFNγ Receptor Phosphopeptide

Colo-205 homogenates were preincubated either in the absence (closed circle) or presence (open square) of IFNγ receptor α chain-derived 436–446 phosphopeptide at concentrations of 0.048, 0.24, 1.2, 6.0, 30, 150 μM. Homogenates were then incubated in the presence of IFNγ for 5 min at 37°C. Extracts were prepared by adding stop buffer containing the IFNγ receptor 436–446 phosphopeptide at concentrations that corresponded to each experimental condition. Stat1 DNA binding activity was assessed by EMSA using a ^{32}P-labeled oligonucleotide probe derived from the GRR of the FcγRI gene promoter. Gel shift complexes were quantitated using a Molecular Dynamic Phosphoimager and plotted as a percentage of Stat1 binding in the absence of phosphopeptide.

436–444 peptide did not affect Stat1 DNA binding activity when added before or after IFNγ treatment (data not shown). Moreover, Stat1 homodimers (formed by coexpressing Stat1 and Jak-1 in cells) were unable to be coprecipitated by biotinylated phosphorylated 436–447 IFNγ receptor peptide (data not shown). Taken together, these results suggest that the Stat1 homodimer is held together with sufficient avidity that its component SH2 domains are unavailable for binding external phospholigand sites. Thus, Stat1 homodimers that form as a result of dissociation of phosphorylated Stat1 monomers from activated receptor docking sites are unlikely to rebind to their receptor tethers.

Discussion

Herein we demonstrate that purified latent Stat1 binds directly to a tyrosine-phosphorylated IFNγ receptor α chain intracellular domain sequence that is known to play a critical role in mediating IFNγ-dependent Stat1 recruitment and induction of biologic responses. Using surface plasmon resonance and immunoprecipitation technologies, we establish the affinity of the interaction between a Stat protein and its physiologic tyrosine-phosphorylated binding site (K_D = 137 nM), demonstrate the specificity of the interaction, and show that the key receptor amino acids that contribute most to the formation of the Stat1 docking site are a phosphotyrosine at position 440, an Asp at position 441, and a His at position 444. We show that Stat1 displays an ordered ability to interact with other Stat proteins. Finally, we suggest that homodimerization of phosphorylated monomeric Stat1 molecules that dissociate from closely juxtaposed receptor docking sites may be a mechanism that favors accumulation of activated Stat1 in the cytosol, thereby making it available for nuclear translocation. We propose that the interactions that occur in cytokine-treated cells between Stat proteins and their respective tyrosine-phosphorylated ligands is an ordered process that is governed in an affinity-driven manner.

The affinity and characteristics (including the rapid dissociation rate) of the interaction between Stat1 and its phosphorylated IFNγ receptor docking site are comparable to that reported for other SH2 domain-containing proteins and model high affinity phosphotyrosine-containing 9–12 aa peptide ligands (K_D = 50–110 nM; Panayotou et al., 1993; Marengere et al., 1994). Importantly, it has been established that 12 aa phosphopeptides are suitable probes for monitoring the binding affinity and specificity of SH2 domain interactions and that longer peptides offer no significant advantage (Piccione et al., 1993). One particularly novel aspect of our current study is that instead of using model phosphopeptides that are selected strictly on the basis of SH2 domain binding affinities, we have employed phosphopeptide sequences that are based on physiologically important regions of authentic Stat1 ligands. The biologic relevance of our observations is supported by our previous biological studies. These analyses established that Y440 is the physiologically important substrate of the IFNγ activatible tyrosine kinase(s) and becomes rapidly (15 s) phosphorylated following addition of IFNγ to cells (Greenlund et al., 1994); phosphorylated Y440, together with D441 and H444, are obligatorily required for IFNγ-dependent Stat1-mediated signaling in cells (Farrar et al., 1992); and a peptide containing phosphorylated Y440, D441, and H444 blocked IFNγ-dependent Stat1 activation in cell homogenates (Greenlund et al., 1994). Thus, the conclusions reached in this current report reflect the merger of both biochemical and biological data.

Using altered phosphopeptide ligands derived from functionally inactive IFNγ receptor α chain point mutants, we established that the amino acids in the receptor docking site at positions +1 and +4 relative to the phosphotyrosine contribute significantly to the affinity of the Stat1–receptor interaction. Ala substitution of either D441 or H444 led to a 25- to 40-fold reduction in Stat1 binding affinity, while Ala substitution of K442 or P443 did not significantly affect the affinity of Stat1 peptide binding. This data correlates with our previous demonstration that Ala substitution of D441 or H444 within the intact receptor α chain ablated signaling activity of the mutant receptor protein (Farrar et al., 1992; Greenlund et al., 1994) and confirms the distinct topographical orientation of the specificity-determining residues in the Stat1 SH2 domain ligand compared with typical Src family SH2 domain ligand (i.e., the specificity-conferring residues occur at positions +1 and +3 relative to the phosphotyrosine; Songyang et al., 1993). Taken together, these results suggest that Stat1 must display a minimal binding affinity to its tyrosine-phosphorylated substrate before Stat activation can occur

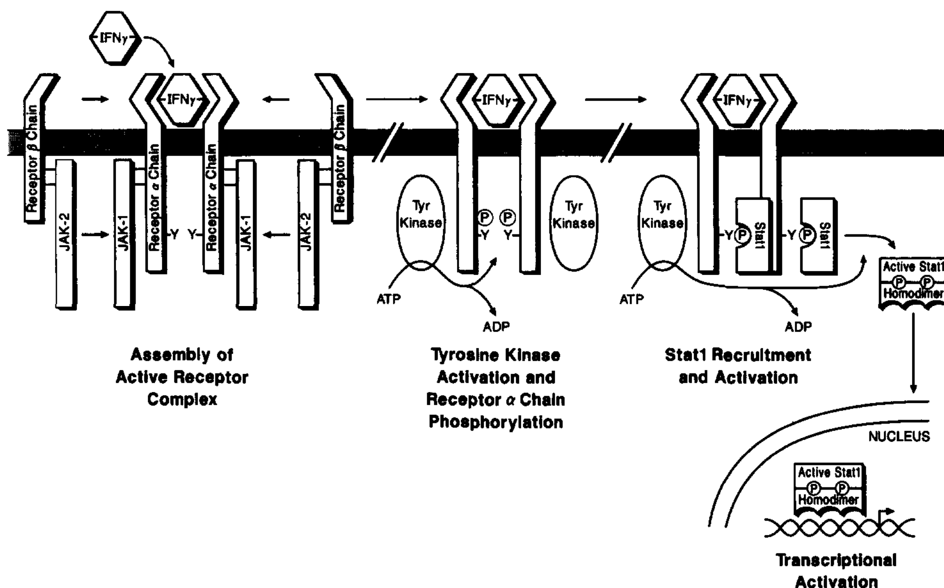

Figure 7. Model of Human IFNγ Signal Transduction

In an unstimulated cell, the human IFNγ receptor α and β polypeptides carry distinct Jak kinases in inactive form (Müller et al., 1993; Watling et al., 1993) but are not preassociated with one another. Jak-1 constitutively associates with the receptor α chain intracellular domain (Igarashi et al., 1994) through an LPKS sequence (receptor residues 266–269; Greenlund et al., 1994; D. H. Kaplan and R. D. S., unpublished data). Jak-2 constitutively associates with the receptor β chain by binding to a β chain PPSIPLQIEEYL sequence (residues 263–274, E. A. Bach and R. D. S., unpublished data). Signal transduction through the IFNγ receptor is initiated following binding of a IFNγ homodimer (Ealick et al., 1991) to two IFNγ receptor α chains, thereby inducing α chain dimerization (Fountoulakis et al., 1992; Greenlund et al., 1993). The receptor β chain then associates with the ligand–receptor α chain complex, thereby bringing the intracellular domains of the receptor polypeptides and the kinases that are associated with them into close juxtaposition. In the complex, the Jak kinases transactivate one another and the activated enzymes then phosphorylate the critical tyrosine 440 residues in each of the dimerized IFNγ receptor α chain intracellular domains. This event forms on the dimerized receptor polypeptides two moderately high affinity, specific, and juxtaposed docking sites for latent Stat1. Stat1 molecules then bind directly to each phosphorylated receptor α chain via the Stat1 SH2 domains (Greenlund et al., 1994; Heim et al., 1995) and are thereby brought into close proximity with the activated receptor-associated tyrosine kinases. Stat1 is subsequently activated by phosphorylation of a specific tyrosine residue (Y701) (Shuai et al., 1992, 1993a, 1993b; Müller et al., 1993; Watling et al., 1993; Silvennoinen et al., 1993). The rapid dissociation of the Stat1–receptor complexes, which are in close proximity with one another, favors the in situ formation of Stat1 homodimers, which are stabilized by reciprocal SH2 domain binding (Shuai et al., 1994). This event thereby releases Stat1 from its receptor tether allowing the activated complex to translocate to the nucleus and induce gene transcription (Shuai et al., 1992).

and that this affinity is governed by amino acids in particular downstream locations from the phosphotyrosine. The interaction of Stat1 with the tyrosine-phosphorylated Stat1 sequence is of even lower affinity than that of the receptor mutants and, therefore, we predict that Stat1 is unlikely to be recruited by a receptor-associated tyrosine-phosphorylated Stat1 molecule. However, since the local concentration of Stat1 at the membrane is unknown, we cannot formally rule out this possibility at the current time.

Two additional mechanisms concerning IFNγ-induced Stat1 activation that need to be defined are the mechanism underlying the exclusive generation of Stat1 homodimers in IFNγ-treated cells and the process by which activated Stat1 is released from its receptor docking site. We now know that IFNγ induces receptor α chain dimerization (Fountoulakis et al., 1992; Greenlund et al., 1993); activation of receptor-associated tyrosine kinase(s) (Müller et al., 1993; Watling et al., 1993); formation of juxtaposed Stat1 docking sites on the dimerized receptor α chains (Dighe et al., 1993; Greenlund et al., 1994); and recruitment and tyrosine phosphorylation of Stat1 (Shuai et al., 1992, 1993a; Greenlund et al., 1994). The formation of IFNγ-induced Stat1 homodimers appears to be a reflection of the ligand-induced dimerization of Stat1 binding sites on the IFNγ receptor α chain, an event that would bring two receptor-associated phosphorylated Stat1 monomers into close proximity with one another. The experimental support for this concept is derived from the observation that human or murine IFNγ receptor α chains containing point mutations at the functionally critical Y440 (human) or Y420 (murine) residues act as dominant-negative mutants when overexpressed in homologous cells (Dighe et al., 1993). Importantly, IFNγ responsiveness can be restored in the inactivated cells by expressing in them the wild-type receptor. These observations thus indicate that the dominant-negative mutants function to prevent formation of dimerized Stat1 binding sites and are not acting by sequestering important components of the IFNγ signaling pathway. The observations reported herein demonstrate that Stat1 rapidly and reversibly dissociates from its receptor binding site and that Stat1 homodimers display decreased ability to interact with the phosphorylated receptor docking site. These observations predict that once the juxtaposed phosphorylated Stat1 monomers dissociate from the receptor they form high avidity homodimers stabilized by reciprocal SH2 domain–phosphotyrosine interactions and therefore

are unable to rebind to the receptor because they lack unoccupied SH2 domains. A proposed model for Stat1 activation by the ligated IFNγ receptor is presented in Figure 7.

In the case of cytokine receptors that produce Stat1 containing heterodimers, a similar scenario can be envisaged except that distinct binding sites for each Stat protein would have to be present on either unique receptor subunits or on an individual receptor polypeptide. Based on the BIAcore binding data, it is predicted that Stat1 would not be recruited by receptor-associated phosphorylated forms of Stat3, Stat4, Stat5, or Stat6 because Stat1 binds to these other phosphorylated ligand sites with an affinity that is significantly below the minimal affinity required for Stat1 activation. This observation explains why Stat1 is not activated by every cytokine receptor. However, it is of significant interest that Stat1 binds to the Stat2-derived phosphopeptide with an affinity that is intermediate between the active IFNγ receptor docking site sequence and the inactive DA or HA mutant sequences. This observation predicts that IFNα receptor-associated tyrosine-phosphorylated Stat2 might act as an effective docking site for Stat1. In fact, recent data utilizing mutant cell lines lacking Stat1 or Stat2 supports this possibility. In the U3 cell line lacking Stat1, IFNα induced the tyrosine phosphorylation of Stat2 (Improta et al., 1994). In contrast, IFNα was unable to induce tyrosine phosphorylation of Stat1 in the Stat2-deficient U6A cell line (Leung et al., 1995). These observations led Leung et al. (1995) to propose that Stat2 might indeed serve as the adapter protein for Stat1 recruitment by IFNα.

The generality of these observations are supported by the work of three other groups as well as separate studies by ourselves. Hou et al. (1994) showed that a purified activated Stat6 homodimer could be dissociated by high concentrations of specific interleukin-4 (IL-4) receptor intracellular domain phosphopeptides containing tyrosine residues 578 or 606. Although these results suggested that phosphotyrosine containing receptor intracellular domain sequences were capable of interacting with purified dimeric Stat6 complexes, they were distinct from the IFNγ receptor studies because the functional importance of the two tyrosine-containing IL-4 receptor sequences has not yet been established and the phosphopeptide binding ability of latent monomeric Stat6 was not assessed. Subsequently, Stahl et al. (1995) showed that Stat3 activating ability could be transferred to the erythropoietin receptor (which does not normally support activation of Stat3) by transfer to the intracellular domain of the receptor of particular tyrosine-containing sequences from the gp130 component of the IL-6/LIF/CNTF receptor family (that has been shown to activate Stat3). Moreover, we have recently identified two Stat3 binding sites located in tandem in the ligand-binding chain of the IL-10 receptor and have confirmed their functional role in manifesting IL-10-dependent cellular responses (R. Weber-Nordt, A. C. G., J.K. Riley, K. W. Moore, J. E. Darnell, Jr. and R. D. S., submitted). Importantly, these sequences were shown to interact with Stat3 but not Stat1 demonstrating Stat specificity for unique receptor sequences. Finally, Heim et al. (1995) recently demonstrated that Stat recruitment by specific cytokine receptors is determined by the specificity of the SH2 domain of the Stat protein. Replacement of the SH2 domain of Stat2 (the family member utilized by the IFNα receptor but not the IFNγ receptor) with the Stat1 SH2 domain led to Stat2 activation by the IFNγ receptor.

Thus, taken together these studies establish the key role that tyrosine-phosphorylated cytokine receptor intracellular domain sequences play in the specific recruitment of particular Stat proteins. As a result of this specific and direct binding and the subsequent affinity-driven interactions of the recruited Stat proteins, cytokines manifest the ability of inducing expression of the genes that ultimately lead to development of cytokine-specific cellular responses.

Experimental Procedures

Stat1 cDNA Preparation

The human Stat1 cDNA was cloned by polymerase chain reaction using a cDNA library prepared from human Colo-205 cells. The resulting cDNA was sequenced using the Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, California) and corresponded to the published sequence (Schindler et al., 1992a).

A 10 residue His tag was engineered onto the amino terminus of Stat1 by a two-step polymerase chain reaction utilizing the pET 16b expression vector (Novagen, Madison, Wisconsin) as a template encoding the His tag. The His-tagged Stat1 cDNA was subsequently cloned into the vaccinia virus recombination vector, PTM3, which was provided by Drs. R. Mercer and K. Grindstaff (Washington University School of Medicine, St. Louis, Missouri and Stanford University, Palo Alto, California, respectively) which places Stat1 under the transcriptional control of the T7 promoter.

The cDNA encoding murine Jak-1 was a gift from Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan). A 10 residue His tag was also engineered onto the carboxyl terminus of Jak-1. The Jak-1 cDNA was subsequently cloned into the PTM3 plasmid.

Generation of Recombinant Stat1 Containing Vaccinia Virus

Wild-type vaccinia virus, VTF7-3 (recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase), and the CV-1 and BSC-40 cell lines were provided by Drs. R. Mercer and K. Grindstaff. Homologous recombination of the PTM3–Stat1 vector into wild-type vaccinia virus was achieved by infecting CV-1 with wild-type vaccinia virus and subsequently transfecting into the infected cells the PTM3–Stat1 vector, as described by Earl et al. (1994). Selection and purification of the recombinant virus was achieved using published protocols (Earl et al., 1994). Recombinant Jak-1 virus was plaque purified and amplified in a manner analogous to the recombinant Stat1 virus.

For large scale preparation of recombinant Stat1, BSC-40 cells were coinfected with VTF7-3 and recombinant Stat1 vaccinia viruses and cultured for an additional 12 hr. Cells were removed from plates by scraping, pelleted by centrifugation, and resuspended to 3.3×10^7 cells/ml in homogenization buffer (50 mM Tris [pH 7.5] containing 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na orthovanadate, 2 mM β-mercaptoethanol). Cells were disrupted by 10 strokes in a steel dounce. Sodium chloride was added to the homogenate at a final concentration of 150 mM, and the homogenate centrifuged for 10 min at 15,000 rpm in a Beckman JA-20 rotor. The supernatant was removed and the recombinant Stat1 protein purified using NTA Ni^{2+} Sepharose (Qiagen, Incorporated, Chatsworth, California) according to the instructions of the manufacturer. Purified Stat1 was dialyzed against 10 mM Tris (pH 6.8), 150 mM NaCl, and 0.5 mM DTT and the protein concentration of the final preparation was determined using the BioRad protein assay (BioRad, Richmond, California). Purified Stat1 (1 mg) was routinely obtained from 6×10^8 virus-infected BSC-40 cells. The preparation was analyzed by SDS–PAGE (Laemmli, 1979) and Coomassie brilliant blue R-250 (BioRad) staining. Western blot analysis was performed on the purified preparation using rabbit sera directed against amino acid 2–66 of Stat1 (29132), amino acid 598–705 of Stat1 (2991) (both of these antisera

were provided by Dr. C. Schindler, Columbia University, New York, New York), and the carboxy-terminal 36 amino acids of Stat1 as described previously (Greenlund et al., 1994).

Generation of Hamster MAbs Specific for Stat1

The Stat1-specific MAbs ATO-1D6 and ATO-2F5 were generated in Armenian hamsters as previously described (Sheehan et al., 1995) following four immunizations with 25 μg of purified recombinant human Stat1. Hybridoma cultures were screened by enzyme-linked immunosorbent assay (ELISA) using immobilized recombinant Stat1. ATO-2F5 and ATO-1D6 reacted in ELISA with full-length recombinant Stat1, a GST fusion protein encompassing the Stat1 SH2 domain (Stat1 residues H557–K668), but not either GST or GST fusion proteins containing other Stat1 molecular regions. These antibodies immunoprecipitated and Western blotted recombinant and natural latent human and murine Stat1 but did not react with activated dimerized Stat1 derived from IFNγ-treated cells.

In Vitro Activation of Stat1

Purified recombinant Stat1 (1 μg) was incubated with 1 μg of purified recombinant Jak-1 in buffer containing 30 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 200 μM ATP in a volume of 25 μl at 23°C for 30 min. We then subjected 14 μl of the reaction to EMSA using a ^{32}P-labeled oligonucleotide probe derived from the γ response region (GRR) of the FcγRI gene promoter as previously described (Pearse et al., 1993; Greenlund et al., 1994).

Peptide Synthesis

The synthesis of the biotinylated 436–447 IFNγ receptor α chain–derived phosphopeptide (TSFGY-*PO₄*DKPHVLV), the biotinylated nonphosphorylated 436–447 peptide, and the biotinylated 458–469 phosphopeptide (SLIGY-*PO₄*RPTEDSK) was previously described (Greenlund et al., 1994). Synthesis of nonbiotinylated phosphopeptides was performed on an Applied Biosystems 432A Personal Peptide Synthesizer using 75 μmol FMOC–phosphotyrosine obtained from Advanced ChemTech (Louisville, Kentucky) and a Synergy empty column kit (Applied Biosystems). All other peptide synthesis and processing was according to the protocol of the manufacturer. Phosphopeptides were analyzed and purified as described (Greenlund et al., 1994). Amino acid sequences of the peptides used in this study are listed in Table 1.

Precipitation of Stat1 with the Biotinylated Phosphopeptides

Biotinylated peptide (100 nM) (436–447 peptide, 436–447 phosphopeptide, or 458–469 phosphopeptide) was incubated 12 hr at 4°C with 10 nM purified recombinant Stat1 in 1 ml of 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 0.5 mM DTT, and 0.5% NP-40. UltraLink immobilized Neutravidin Plus (40 μl) on 3M Emphaze Biosupport Medium AB 1 (Pierce, Rockford, Illinois) was added and the incubation continued for an additional 1 hr at 4°C. The biosupport was then washed and precipitates analyzed by SDS–PAGE and Western blotting with rabbit anti-Stat1 carboxyl terminus serum as described (Greenlund et al., 1994).

In some experiments, purified Stat1 (10 nM) was pretreated for 5 hr at 4°C with different amounts of purified anti-Stat1 ATO-2F5 MAb, irrelevant species-matched H22 MAb (Schreiber et al., 1985), or 15 μl of either normal hamster sera or rabbit antisera directed against the carboxy-terminal 36 aa of Stat1. Biotinylated IFNγ receptor-derived 436–447 phosphopeptide (33 nM) was then added and the reaction mixtures processed as described above.

Analysis of Stat1 Binding to the IFNγ Receptor α Chain 436–447 Phosphopeptide Using Surface Plasmon Resonance

The Pharmacia BIAcore Biosensor employs the phenomenon of surface plasmon resonance to monitor the refractive index change in a flow cell due to the binding of macromolecules to immobilized ligand (BIAcore User Manual, Pharmacia, Piscataway, New Jersey). The accumulation of mass on the flow cell surface effects the refractive index of the surface, thereby effecting the intensity of the reflected light. Since protein mass determines the refractive index, the change in light intensity is directly proportional to the protein mass bound to ligand.

IFNγ receptor α chain biotinylated 436–447 phosphopeptide was immobilized on a streptavidin sensorchip surface (SA-5, Pharmacia) by injecting 10 μl of 20 nM peptide in 10 mM HEPES buffer (pH 7.5) containing 500 mM NaCl at a flow rate of 5 μl/min. Phosphopeptide coupling to the sensorchip surface was monitored using the anti-phosphotyrosine MAb, PY-20. Biotinylated forms of IFNγ receptor-derived nonphosphorylated 436–447 peptide and phosphorylated 458–469 peptides were immobilized in a similar manner.

To determine the equilibrium dissociation constant (K_D) describing the interaction of Stat1 with the immobilized 436–447 phosphopeptide, purified Stat1 preparations at concentrations ranging from 38–200 nM in 10 mM HEPES buffered physiologic saline (pH 7.4) containing 3.4 mM EDTA and 0.005% P-20 surfactant (Pharmacia) were injected over the phosphopeptide surface at a flow rate of 3 μl/min. The steady-state equilibrium response (R_{eq}) determined using the BIAevaluation 2.1 software (Pharmacia) was subjected to Scatchard analysis to calculate the K_D. The kinetic on and off rate constants were calculated using surface plasmon resonance binding data from the association and dissociation phases of the reaction using the BIAevaluation 2.1 software.

The K_D describing the interaction of Stat1 with the soluble IFNγ receptor 436–446 phosphopeptide in solution was also determined using the BIAcore. Purified Stat1 (0.5 μM) was incubated with the soluble IFNγ receptor 436–446 phosphopeptide at concentrations ranging from 0.016–10 μM for 30 min at 10°C. The mixtures were then injected over a sensorchip surface containing immobilized IFNγ receptor α chain 436–447 phosphopeptide. The K_D was calculated based on the initial binding rate of Stat1 in the presence of the various phosphopeptide concentrations using the BIAevaluation 2.1 software solution affinity model (BIAcore User Manual).

Competition Binding Analysis Using the BIAcore Biosensor

Stat1 (0.5 μM) was incubated with various concentrations of different nonbiotinylated phosphopeptides. The mixtures were then injected onto a sensorchip surface to which the biotinylated 436–447 phosphopeptide was immobilized. To eliminate refractive index changes due to differences in solution composition, a report point was monitored 20 s after completion of the injection. Stat1 binding in the presence of phosphopeptide was then calculated as a percentage of total Stat1 binding in the absence of competitor. Nonspecific association was determined as the response (RU) in the presence of 10 μM 436–446 phosphopeptide (less than 2% of total binding).

Inhibition of Stat1 DNA Binding Activity Using a Cell-Free Activation Assay

Cellular homogenates were prepared from Colo-205 cells by dounce homogenization as previously described (Greenlund et al., 1994). Aliquots (50 μl) were incubated for 45 min on ice either in the absence of peptide or the presence of different concentrations of the IFNγ receptor α chain–derived 436–446 phosphopeptide. The reactions were prewarmed for 2 min at 37°C, then treated with 1900 IRU of IFNγ (which was provided by Genentech, Incorporated, South San Francisco, California) for 5 min. Stop buffer (450 μl) (Greenlund et al., 1994) containing different concentrations of the 436–446 phosphopeptide was added to each reaction that was preincubated with peptide or without peptide prior to IFNγ treatment. Samples were vortexed 3 s, incubated on ice for 5 min, and subjected to centrifugation at 12,000 × g for 5 min at 4°C. Supernatants were removed and the incubation continued an additional 45 min on ice. Of each reaction, 14 μl (5 μg) was then subjected to EMSA using the GRR oligonucleotide probed as described above. Binding of Stat1 to the oligonucleotide probe was quantitated using a Molecular Dynamics Phosphoimager (Sunnyvale, California).

Acknowledgements

The authors are grateful to Dr. P. Allen and Mr. D. Donermeyer, Department of Pathology, Washington University School of Medicine, for help in generating peptides and Drs. K. Grindstaff and R. Mercer, Department of Cell Biology and Physiology, Washington University School of Medicine, for helping us establish the Vaccinia virus expression system in the laboratory. The authors also wish to thank Dr. J. Darnell, Rockefeller University; Dr. S. McKnight, Tularik, Incorporated; and Dr. C. Schindler, Columbia University College of Physicians and

Surgeons for reagents and helpful discussions. This work was supported by a grant from the National Institutes of Health (CA43059).

Received April 25, 1995; revised May 12, 1995.

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