A Single Residue Modulates Tyrosine Dephosphorylation, Oligomerization, and Nuclear Accumulation of Stat Transcription Factors*

The NH2 terminus of Stat proteins forms a versatile protein interaction domain that is believed to use discrete surfaces to mediate oligomerization and tyrosine dephosphorylation of Stat dimers. Here we show for Stat1 and Stat5a/b that these interfaces overlap and need to be reassigned to an unrelated region of the N-domain. Unexpectedly, our study showed for Stat1 that defective oligomerization of DNA-bound dimers was associated with prolonged interferon-induced nuclear accumulation. This uncoupling of DNA binding and nuclear retention was explained by the concomitant dephosphorylation deficiency that both Stat1 and Stat5a/b have in common and that for Stat1 was due to defective dephosphorylation by the phosphatase TC45. Furthermore, diminished N-domain-mediated oligomerization affected transcriptional activation by both Stat1 and Stat5a/b in a promoter-specific manner. DNA binding analysis indicated that oligomerization of Stats on DNA may be common, irrespective of the presence of multiple canonical binding sites. Accordingly, transcription from promoters with only a single discernable γ-activated sequence site was negatively effected by reduced tetramerization. Thus, these results indicate that defective oligomerization cannot generally be compensated for by enhanced tyrosine phosphorylation and prolonged nuclear accumulation. In addition, these data clarify the role of DNA binding in nuclear retention of Stat1.

The signal transducer and activator of transcription (Stat) proteins comprise an evolutionary conserved family of transcription factors (1). Canonical signaling through the Janus tyrosine kinase (Jak)/Stat pathway begins at the cell membrane with the engagement of cytokines with their cognate receptor (2). This triggers the autophosphorylation on tyrosines of non-covalently attached Jak kinases, which also phosphorylate signature tyrosine residues in the intracellular receptor tails thus providing phosphotyrosine docking sites for the Stat SH2 domain (3). The bound Stat monomers detach from their receptor docking sites after phosphorylation of a single tyrosine residue at their COOH terminus and form high avidity reciprocal homo- or heterodimers (4–6). This sequence of events is commonly referred to as “Stat activation” and within minutes triggers the accumulation of Stat dimers in the nucleus due to their inability to leave this compartment (7). Here they can bind to palindromic DNA recognition sites (GAS) and directly induce transcription (8).

The products of seven different Stat genes and various splice variants thereof can be found in mammalian cells. Gene activation experiments using homologous recombination have revealed that the most striking phenotypes are associated with signal transduction by particular cytokines. Stat1 is required for interferon-α (IFNα) and IFNγ signaling, and mice and humans lacking Stat1 are extremely sensitive to viral and microbial infection (9–11). The two highly related Stat5a and Stat5b proteins (~96% sequence identity) are activated by a wide range of different cytokines such as interleukin 2 (IL-2), IL-7, prolactin, and growth factors. Accordingly, gene ablation experiments uncovered mammary gland defects, growth abnormalities, and an absent IL-2 response (12–14).

Stat proteins consist of a large core domain including the DNA binding module (15) and the SH2 domain responsible for dimer formation (5). The core is connected through a proteolytically cleavable peptide to the smaller N-domain, which engages in a multitude of protein-protein interactions. The N-domain mediates cooperative DNA binding of Stat dimers (16–20) and controls their tyrosine dephosphorylation (21, 22). The structure of the isolated N-domain reveals an all-helical hook-like appearance (23). In solution the N-domain forms stable dimers; however, two alternate binding surfaces are formed in the crystals. Recent reevaluation of the crystal-packing has indicated that the initial assignment of one of these surfaces as being responsible for cooperative DNA binding is probably incorrect (24). An unrelated region of the N-domain has previously been identified by mutagenesis to be important for the dephosphorylation of Stat1, as mutation of an invariant residue caused severe hyperphosphorylation (21). However, this mutation affects the salt bridges that interlink the helices that make up this domain and thus may impair the structural integrity of this domain. Therefore, at present the physiological interfaces that modulate cooperative DNA binding and tyro-
Diphosphorylation and Oligomerization of Stats

EXPERIMENTAL PROCEDURES

Cell Culture and Antibody Microinjection—Human HeLa S3, U3A, and 293T cells were grown as described previously (25). Unless otherwise noted, other conditions were as follows. Unless noted otherwise, treatment of cells was with 2 ng/ml (hu) IL-2 (Roche), 100 ng/ml IL-1 δ (R&D Systems), 500 IU/ml human interferon γ (Roche), 500 ng/ml staurosporine (Sigma), 50 μM MG132 (Calbiochem). Antibody microinjection into HeLa cells transiently expressing GFP-tagged Stat1 variant proteins was done with monomeric GFP antibody 2A3 as described previously (7, 26).

Plasmids, Transfections, and Luciferase Assays—pCIStat5a, pCIStat5b, pStat1-GFP, and pStat1-DNA−DNA− have been described previously (7, 17, 25). Site-specific mutagenesis of these plasmids according to manufacturer’s instructions (QuickChange, Stratagene; Morph site-specific mutagenesis, 5 Prime — 3 Prime, Inc., Boulder, CO) generated the respective N-domain mutants (F77A for Stat1, F81A for Stat5) (28). The mutations were confirmed by DNA sequencing. Additionally for use in reporter gene assays, the cDNAs of Stat5a and Stat5b were cloned into pcDNA3. For IL-2 receptor reconstitution transfection assays, 4 × 10⁴ 293T cells were transfected with the following DNAs: 2 μg of IL-2 receptor β, 0.5 μg of γc, 0.25 μg of Jak3, and 0.5 μg (for electrophoretic mobility shift assays) or 10 ng (for luciferase assay) of each of the WT Stat5 or F81A mutant expression plasmids. Additionally for the luciferase assay, 1 μg of positive regulatory region III (PRRIII-E1b) or M10-E1b-luciferase reporter constructs (17) and 0.5 ng of the transcription control plasmid, pRLTK-luciferase, was added. The total amount of DNA transfected was kept at a constant amount of 10 μg with empty pCI vector (Promega). Twenty-four hours after transfection, when appropriate, cells were split into two, and 48 h after transfection one set was left untreated and the other was treated with 2 nM IL-2 for 30 min (or IL-2 receptor stimulation with new medium containing appropriate chemicals. Cells were washed once with ice-cold phosphate-buffered saline and whole cell lysates were prepared by boiling the cell pellet in 200 μl of SDS sample buffer for 5 min. Ten μl of each sample was loaded on 8% SDS-PAGE gels. Western blotting, histone deacetylase (HDAC) inhibition, and FRAP were done as described previously (25) using a phosphotyrosine-specific Stat5 antibody (Tyr694, eStat5-P) or a pan-Stat5 antibody (both from Transduction Laboratories) or a Tyr701 phosphospecific Stat1-P (Cell Signaling) or Stat1 antibody (Santa Cruz Biotechnology). In vitro diphosphorylation reactions were performed for 60 min with 0.5 nm pBacPAK8 (Clontech) that was not treated with 2 μl of recombinant TC45 (Sigma) and the indicated amount of unphosphorylated Stat1 as described previously (7). Unphosphorylated Stat1 was added at a concentration of 0.5, 5, or 20 nM. The mixture was resolved by SDS-PAGE after boiling in SDS sample buffer.

Expression and Purification of Recombinant Proteins—Recombinant Stat1a and His-tagged Stat5a proteins were produced in baculovirus-infected insect cells and purified as described previously (7, 28). Stat5a was tyrosine-phosphorylated by an endogenous kinase during expression in the insect cells (28). In vitro tyrosine phosphorylation with epidermal growth factor receptor and isolation of phosphorylated Stat5a were done as described previously (7). For production of Stat1F77A, the correctly mutated cDNA was excised (NotI) from pcDNA-Stat1-F77A (Stat1 was tagged with baculovirus transfer vector Ad5, kindly provided by Dr. P. B. Tonegawa). For production of recombinant Stat5aF81A the wild type Stat5a cDNA sequence, which was cloned into the baculovirus transfer vector pBacPAK8 (Clontech) was mutated by site-directed mutagenesis at the appropriate residue and confirmed by DNA sequence analysis. Both Stat5a and Stat5aF81A were confirmed to be tyrosine-phosphorylated by Western blot analysis using anti-phospho-Stat5 antibody. Truncated TC45 was produced in bacteria and purified as described previously (7).

Fluorescence Microscopy and Fluorescence Recovery after Photo-bleaching (FRAP)—Fluorescence microscopy and FRAP were performed as described previously (7).

RT-PCR—RT-PCR analysis was done as described previously (26) with USP cells stably expressing Stat1 or Stat1F77A and the following primer pairs: IRP-1 (GenBank™ accession number NM_002198), 5′-TAAGACACCTGGCTTAGAG and 5′-AAGGATCTCGTATCACTCG; LMP-2 (GenBank™ accession number U10205), 5′-GGGCGTTGTGATGCGTGGCTGTGATTC and 5′-TCGGGACATGGCTGGCTTTATAT; ISG-15 (GenBank™ accession number NM_051011), 5′-GCGGCGGCGAACAGAATTCGAGGCTGCTC and 5′-CCCCGGAGCAGGAGCCACGGG; NS1 (GenBank™ accession number M24584), 5′-AGGCGCAACAGAGAAGGCTGATAC and 5′-CCGGCTCATAGTACTCCAGGGCTTCATTC.

RESULTS

The Alternate N-domain Interface Mediates Cooperative DNA Binding of Stat1 and Stat5αb—Studies of Stat oligomerization from our and other labs have indicated that a single invariant residue (Trp37 of Stat1) that is located in the crystallographic interface of N-domain dimers affects tetramerization of full-length Stats (17, 20, 23). However, in vitro studies with the isolated N-domain demonstrated that mutation of Trp37 causes destabilization and degradation of the N-domain (24), and the Stat5W37A mutants were previously found to be expressed less efficiently compared with their WT counterparts in either a baculovirus expression system or in IL-2 receptor-reconstituted 293T cells (17, 28). The crystal packing of the isolated N-domain also revealed another mode of N-domain interactions not involving Trp37 (24). Therefore, we mutated in the full-length Stat molecule a conserved hydrophobic residue on the surface of the N-domain that was demonstrated to affect dimerization of N-domains (Phe97 → Ala in Stat1 and Phe91 → Ala in Stat5αb, respectively). As is shown in Fig. 1, A and B, the resulting Stat1 and Stat5αb mutants were expressed well and no indication of structural instabilities was detected by SDS-PAGE and Western blotting. However, expression of the Stat5αF81A mutant was reduced in 293T cells compared with WT (Fig. 1B). Therefore, we produced recombinant Stat5αa protein in baculovirus infected insect cells (Fig. 1C). In these cells, Stat5αF81A was expressed even better than WT. Phosphory-
Fig. 1. Expression and DNA binding of N-domain mutants of Stat1 and Stat5a/b. A, HeLa cells transiently expressing GFP-tagged Stat1WT or F77A mutant were treated with or without IFNγ for 30 min and harvested. Shown are Western blots of whole cell extracts developed with a phospho-Stat1-specific antibody (αStat1-P) and a Stat1-specific antibody. B, Western blot of nuclear extracts (2 μg of protein/lane) from IL-2 receptor-reconstituted 293T cells. The expression of transfected Stat5a or Stat5b was probed with a pan-Stat5 antibody. C, increasing amounts of purified recombinant Stat5aWT or F81A-mutant (125, 250, or 500 ng) were Western blotted and probed with a pan-Stat5 and phospho-Stat5 antibody. D, competition gel-shift assay with cytoplasmic extracts from IFNγ-stimulated reconstituted U3A cells and a single GAS site. Before loading on the gel, the reaction mixture was incubated for the indicated times in the presence of a 750-fold molar excess of unlabeled oligo; alternatively the reaction included a Stat1-specific antibody (S). The positions of dimeric Stat1 (D) and an unspecific band (*) are indicated. E, gel-shift analysis of wild type or mutant Stat5a and Stat5b with a single GAS element (MGF). Phosphorylated Stat5a (125, 250, or 500 ng) purified from insect cells and nuclear extracts (10 ng protein/lane) from untreated or IL-2 treated reconstituted 293T cells expressing Stat5b were used. F, gel-shift analysis of wild type and mutant Stat5a and Stat5b. Left panel, nuclear extracts (10 μg of protein/lane) from IL-2 receptor-reconstituted 293T cells expressing the indicated constructs were incubated with a single high affinity MGF site or the PRRIII probe containing tandem...
ation by an endogenous kinase yielded correctly tyrosine-phosphorylated Stat5a protein (Fig. 1C and Ref. 17), and this material was therefore used in subsequent DNA binding experiments.

The Phe77 and Phe81 mutants of Stat1 or Stat5a/b, respectively, were tyrosine phosphorylated in response to cytokine stimulation of cells and bound to a single optimal GAS site (M67 and MGP, respectively) similar to wild type, as did recombinant Stat5a/P81A (Fig. 1, D, E, and left panel of F). Next, we tested the ability to form tetramers on tandem GAS sites by EMSA analysis. For Stat5 we used an oligonucleotide containing tandemly linked GAS motifs, derived from the IL-2 response element that is contained in both the murine and human genes coding for the α-subunit of the IL-2 receptor, termed PRRIII. We and others (17, 28, 29) have previously shown that binding of endogenous Stat5 to isolated single GAS sites from PRRIII is not discernable, but an oligonucleotide comprising full-length PRRIII binds Stat5a and Stat5b as tetramers (Fig. 1F). Consistent with previous observations (28), tetramerization of Stat5b on PRRIII was weak compared with Stat5a (Fig. 1F, left panel). In contrast to wild type Stat5a/b, mutation of residue Phe81 to Ala caused a loss of tetramer formation as indicated by the inability to bind to the tandemly linked GAS motifs in PRRIII (Fig. 1F, left panel). In addition to nuclear extracts from reconstituted 293T cells, we used purified recombinant Stat5a proteins to explore binding to the PRRIII probe, which confirmed the tetramerization defect of the Stat5a/P81A mutant (Fig. 1F, right panel). EMSA analysis with tandem binding sites was also performed with Stat1WT and Stat1/F77A (Fig. 1G). An artificial construct containing two high affinity GAS sites from the human IRF-1 promoter (5′-TTCCCGGAA) was used in these experiments. Stat1 can bind independently to either site, resulting in both a fast migrating Stat1/DNA complex containing a single Stat1 dimer as well as a slow migrating complex with two Stat1 dimers. When such complexes were challenged for 30 min with a 750-fold molar excess of unlabeled oligonucleotide, the dimeric complexes of both wild type and mutant were displaced. However, the tetrameric complex resisted displacement only with Stat1WT, whereas the respective complex of the F77A mutant was dispelled (Fig. 1G), indicating that adherent dimers of Stat1/F77A interacted only weakly via stabilizing contacts.

To further explore DNA binding and tetramerization of Stat1, we included in our analysis another three 41-mer oligonucleotides. Two of them each contained a single canonical GAS site (5′-TTCCCGGAA), and a second site 11 bp away that was either unrelated (non-GAS; 5′-TCCGTGCTA) or bore little resemblance to GAS sites (GAS-like; 5′-TTATCATTA). Additionally, a 41-mer without sequences resembling GAS elements was used (see “Experimental Procedures”). We used in vitro tyrosine-phosphorylated Stat1 at a concentration of 1 nM for these experiments (which is also the apparent equilibrium constant of the Stat1 DNA-binding reaction; see Ref. 18). The results are shown in Fig. 1, H and I. Expectedly, a fast migrating band of dimeric Stat1 bound to canonical GAS sites was seen (Fig. 1H, lanes 2–5). Strikingly, Stat1 tetramers formed not only with the tandem canonical GAS sites (Fig. 1H, lane 2) but also with the 41-mer oligonucleotides that harbored only a single GAS site (Fig. 1H, lanes 4 and 5). DNA binding of Stat1 was observed even in the complete absence of any GAS resemblance of the DNA, but binding was confined to tetramers (Fig. 1H, lane 6). Of note, tetramerization required the presence of a DNA stretch, as no band indicative of tetramer formation was seen with a short 28-mer oligonucleotide containing a single binding site (Fig. 1H, lane 3). Importantly, the mutant F77A did not form tetramers on DNA where a single high affinity GAS site was adjacent to a GAS-like site (5′-TTATCATTA) (Fig. 1F, compare with Fig. 1G, where oligonucleotides containing tandem canonical GAS sites were used). Thus, N-domain interactions of DNA-bound Stat molecules made tetramerization possible even in the presence of only a single canonical GAS site. In summary, these data revealed that the phenylalanine residues in position 81 of Stat5 and 77 of Stat1 maintain N-domain interactions. We thus conclude that the recently identified alternate interface is physiologically relevant.

Tetramerization Deficiency Is Associated with Prolonged Nuclear Accumulation—The molecular mechanisms underlying nuclear accumulation of Stat1 were elucidated very recently (7). It was shown that Stat1 can leave the nucleus only after its tyrosine dephosphorylation, and DNA binding was identified as the critical regulator of the conversion. Importantly, impaired DNA binding resulted in shortened nuclear retention. Thus, we investigated nuclear accumulation of the tetramerization-deficient Stat1/F77A mutant, expecting diminished nuclear retention due to the negative effect that the mutation exerted on DNA binding. Surprisingly, our expectations were not met, as we observed a markedly prolonged accumulation phase in IFN-γ-stimulated cells (Fig. 2A). Moreover, we observed a distinct insensitivity to pharmacological kinase inactivation, since nuclear accumulation of the mutant persisted also in the presence of staurosporine (Fig. 2B). This is in stark contrast to wild type Stat1, where staurosporine caused a rapid collapse of nuclear accumulation (Fig. 2B). We then employed an antibody microinjection assay to explore the rate of nuclear-cytoplasmic shuttling of GFP-tagged Stat1 during nuclear accumulation (7, 26). It was previously demonstrated that antibody microinjection can cause precipitation of the targeted molecule. In the case of a shuttling protein this lead to the depletion of the respective molecule from the non-injected compartment (26). This is shown in Fig. 2C for Stat1WT during its nuclear accumulation in interferon-γ stimulated cells. Strikingly, the nuclear retention time during IFN-γ-induced accumulation was considerably extended for mutant Stat1, as it was impossible to achieve its clearance from the nucleus after cytoplasmic microinjection of a GFP antibody (Fig. 2C).

According to our previous results the intranuclear mobility of Stat1 is inversely correlated with the strength of DNA binding (7). We employed FRAP analysis to examine this also for Stat1/F77A (Table I). Expectedly, both the mutant and wild type Stat1 were highly mobile during nuclear accumulation,
and the mobility was reduced only modestly by the inactivation of tyrosine phosphatases. Notably, though, the mutant was even less sensitive to phosphatase inhibition, as its mobility remained significantly higher than wild type (Table I). These results indicated that the prolonged nuclear accumulation period of the mutant Stat1F77A was uncoupled both from DNA binding capacity and reduced intranuclear mobility. The mutant thus differed from the nuclear retention mutants described to date (7). We therefore tested whether the loss of nuclear accumulation that was previously observed with the DNA binding mutant Stat1-DNAminus, which has lost both sequence-specific and -unspecific DNA binding (7), could be reversed by the additional mutation of Phe77. As is shown in Fig. 2D, this was indeed the case, as the F77A mutant of Stat1-DNAminus displayed completely restored nuclear accumulation after 60 min of IFNγ stimulation. These observations can be reconciled with the model of Stat nucleocytoplasmic shuttling if we propose a dephosphorylation defect to be part of

![Cell images](https://example.com/cell_images.png)
The formation of Stat tetramers and higher order oligomers is stabilized by proteasome inhibitors such as MG132 (Fig. 3D). Thus, together these results demonstrate that only natural GAS sites were sensitive to weakened N-domain interactions.

We also tested expression of endogenous IFNγ-responsive genes by semiquantitative RT-PCR (Fig. 4E). Of the seven genes tested, only LMP-2 expression was unaffected by the N-domain mutation of Stat1, whereas the others showed a strongly diminished IFNγ-responsiveness in cells expressing Stat1F77A. Strong effects were seen with the MIG-1, IRF-9, and GBP-1 genes, which had lost their IFNγ responsiveness in cells expressing Stat1F77A. Thus, together these results demonstrate that only natural GAS sites were sensitive to weakened N-domain interactions.

DISCUSSION

The formation of Stat tetramers and higher order oligomers on DNA results from cooperative DNA binding of Stat dimers that appears to be conserved throughout the Stat family, and

The Interfaces for Dimer-Dimer and Dimer-Phosphatase Interactions Are Overlapping—At first, the kinetics of tyrosine dephosphorylation was investigated in living cells. U3A cells, which do not express endogenous Stat1 (30), or 293T cells that were reconstituted with IL-2 receptor components, were transfected with plasmids expressing Stat1 and Stat5, respectively. Subsequently, the cells were stimulated for 30 min with IFNγ to activate Stat1 or IL-2 to activate Stat5. After this initial stimulation period the cytokines were removed, and the cells were further incubated in the absence or presence of the kinase inhibitor staurosporine. As is shown in Fig. 3A, Stat1F77A displayed prolonged tyrosine phosphorylation and insensitivity toward staurosporine, which is indicative of a reduced dephosphorylation rate. A similar outcome was observed also for Stat5a and Stat5b (Fig. 3B). Interestingly, in the IL-2 receptor-reconstituted 293T system the kinetics of dephosphorylation of wild type Stat5 proteins is longer (2–4 h) than that observed in lymphoid cells, where it occurs within 1–2 h (Fig. 3B). Nevertheless, mutation of residue 81 also prolonged tyrosine phosphorylation even further, as the phosphotyrosine signal now persisted by proteasome inhibitors such as MG132 (Fig. 3B) (31). Addition of the kinase inhibitor staurosporine at the same time as the proteasome inhibitor cancelled the effect of MG132 and caused rapid dephosphorylation (Fig. 3B). Importantly, the N-domain mutants of Stat5a/b were again refractory to the action of staurosporine and stayed phosphorylated much longer than wild type Stat5. To exclude the trivial explanation that MG132 is simply inactivated by the presence of staurosporine, we examined the well known MG132-sensitive degradation of IκBα after TNFα stimulation of cells (32). Clearly, MG132 was a potent inhibitor of the TNFα-induced degradation of IκBα both in the absence and presence of staurosporine (data not shown). We therefore conclude that the observed proteasome-dependent down-regulation of phosphorylated Stat5 is not the result of direct Stat5 targeting by the proteasome. Rather, during MG132 treatment a constituent of the IL-2 pathway is stabilized by proteasome inhibitors such as MG132 (Fig. 3B). These findings show that Stat1F77A and Stat5F81A are not only tetramerization-deficient but also display defective tyrosine dephosphorylation in vivo.

To examine whether the N-domain mutation directly affected the interaction between Stat dimer and phosphatase, we performed in vitro dephosphorylation assays with purified tyrosine-phosphorylated recombinant Stat1F77A and TC45, a ubiquitously expressed Stat1-specific nuclear tyrosine phosphatase (33). The dephosphorylation rate of the Stat1 variant protein was strongly reduced (Fig. 3C), indicating that this mutant is defective in direct Stat-phosphatase interactions. Thus, the N-domain surface responsible for cooperative DNA binding of Stat1 dimers is also governing interactions with the phosphatase TC45. When we added up to 40-fold molar excess of unphosphorylated Stat1 to the in vitro dephosphorylation reaction, we did not note an influence on the interaction of phosphatase and its substrate (Fig. 3D), which indicates that the dephosphorylation reaction is not subject to product inhibition.

Mutation of the Tetramerization Interface Affects Transcription in a Promoter-specific Manner—Finally the impact of the N-domain point mutation on gene transcription was investigated. Reporter gene assays were used to assess the consequences of decreased tetramer stability and enhanced tyrosine phosphorylation on gene induction. As shown in Fig. 4, the observed effects depended on the promoter construct used. Reporter genes containing optimal GAS sites were transactivated as good as or even better by the mutant Stats than by the respective wild type counterparts (Fig. 4, A–D). For Stat1 a synthetic promoter with three strong GAS sites separated by 10 bp was used (Fig. 4A). However, when natural promoter fragments were tested, transactivation was lower with the mutants as compared with the respective wild type Stat protein. This was exemplified for Stat1 by transient transfection of a luciferase reporter containing the full-length or truncated promoter of the human ICAM-1 gene (Fig. 4B). Besides binding sites for NFκB, AP-2, and Sp1, this promoter harbors a single canonical GAS site (5′-TTCGGGAA) (27, 34).

Analysis of Stat5-dependent transcription was examined in reporter gene assays with WT PRRIII and a PRRIII derivative (M10), in which the natural weak tandem GAS sites were transformed into strong Stat5 binding sites (17). IL-2 inducibility of the wild type PRRIII promoter was lost for both Stat5a and Stat5b mutants (Fig. 4C; note the considerable reporter gene activation by IL-2 even in the absence of Stat5). However, both the Stat5F81A mutants were better than their wild type counterparts at activating transcription from the high affinity M10 promoter (Fig. 4D). Thus, these results demonstrate that only natural GAS sites were sensitive to weakened N-domain interactions.

—S. John, unpublished observation.

Table I

|                | WT         | F77A       |
|----------------|------------|------------|
| Nucleus unstimulated | 19.0 ± 9.6 | 20.4 ± 3.7 |
| Nucleus IFNγ | 19.6 ± 6.9 | 19.6 ± 4.1 |
| Nucleus IFNγ+vanadate | 62.7 ± 2.7 | 43.5 ± 7.6 |

* Indicates statistically significant difference (p < 0.05).

## DISCUSSION

The formation of Stat tetramers and higher order oligomers on DNA results from cooperative DNA binding of Stat dimers that appears to be conserved throughout the Stat family, and
an invariant Trp residue in position 37 of the N-domain has been demonstrated to influence this process (17, 18, 20). However, recent in vitro evidence indicated that mutation of residue 37 might cause global perturbations of the domain structure, and an alternate surface was proposed to mediate Stat oligomerization (24). This study was initiated to distinguish between these possibilities. Our data demonstrate that mutation of a conserved residue located in the alternate surface does not compromise protein stability but causes a severe decrease in tetramer formation of Stat1, Stat5a, and Stat5b without overtly affecting DNA binding of the dimer. During preparation of this manuscript, the same conclusion was reached also for Stat4 (36). Thus, based on these results we propose the alternate dimerization surface of N-domains as physiologically relevant for oligomerization of Stat proteins. Previous data generated with Trp37 mutants most likely reflect the consequences of nonspecific N-domain destabilization rather than specific interface perturbations. Nevertheless, conclusions concerning cooperative DNA binding remain correct, but data generated in vivo, e.g. transcription analyses, should be reconsidered for the same reason. It remains to be seen whether the original interface of N-domain dimers with Trp37 at its center is required for functions other than oligomerization, such as interactions between unphosphorylated Stats.

Our study shows that the interface that organizes cooperative DNA binding is also required for tyrosine dephosphorylation of Stat1 and Stat5, as the oligomerization mutants were refractory to tyrosine dephosphorylation in vitro. By using in vitro dephosphorylation assays with purified proteins, for Stat1 this could be attributed to a loss of dephosphorylation by the phosphatase TC45. Moreover, progression of the Stat1 dephosphorylation reaction was not inhibited by its reaction product, stressing the very different biochemical properties of the N-domain of phosphorylated and unphosphorylated Stat1. Currently, the role of the N-domain in the inactivation of Stat5 is controversial, since the carboxyl-terminal transactivation domain has been implicated in the proteolytic turnover of tyrosine-phosphorylated Stat5 (31, 37). Here, it is demonstrated that stabilization of activated Stat5 by the proteasome inhibitor MG132 is not due to protection from proteolytic degradation or phosphatase attack, as simultaneous treatment with proteasome and kinase inhibitors resulted in rapid dephosphorylation of wild type Stat5 (Fig. 3B). Importantly, the N-domain mutants both resisted dephosphorylation under these conditions. This indicates that the prolonged tyrosine phosphorylation observed after removal of the COOH terminus and in the presence of MG132 is in fact the result of Stat5 hyperphosphorylation and not of defective dephosphorylation. This conclusion is supported by reports showing that proteasome inhibition prevents Jak kinase inactivation and receptor-mediated endocytosis of the interleukin-2 receptor complex (38, 39). The identity of the Stat5 phosphatase is less clear as different studies have indicated different phosphatases, such as SHP-2, PTP-1B, and TC45, to be involved in the dephosphorylation of Stat5 (33, 40–42).

The cytokine-induced nuclear accumulation of Stats requires the presence of the N-domain (22). Mutation of residue 77 in Stat1 and 81 in Stat5a/b did not affect nuclear import after stimulation with cytokines. On the contrary, mutation of phenylalanine 77 of Stat1 even prolonged nuclear accumulation (Fig. 2A). Both its dephosphorylation defect as well as its tetramerization deficiency measurably affected the nuclear accumu-
Fig. 4. Gene activation by N-domain mutants of Stat1 and Stat5a/b. A and B, reporter gene assays with transiently transfected U3A cells and a luciferase gene driven by a triple GAS site from the Ly6E promoter (A) or fragments (pIC1352 or pIC339) from the ICAM-1 promoter (B). Cells were stimulated without (gray bars) or with IFNγ for 6 h (black). C and D, IL-2-reconstituted 293T cells were co-transfected with wild type or mutant Stat5a or Stat5b and a luciferase reporter gene. Stimulation without (gray) or with IL-2 was overnight (black). Luciferase expression was activated from a natural PRRIII promoter (C) or a synthetic M10 element (D). E, left, RT-PCR analysis of endogenous IFNγ- and IFNγ-responsive genes. The assay was performed with U3A cells stably expressing Stat1WT or F77A mutant. Cells were grown for 15 h in Dulbecco's modified Eagle's medium with reduced serum (1%), followed by the addition of serum to 10% and treatment with or without IFNγ or for 4 h. RNA was extracted and reverse transcribed followed by PCR. Expressions of Stat1 and GAPDH are shown as controls. Shown are the ethidium bromide-stained PCR products (no RT, no reverse transcription before PCR). Right, quantification of the ethidium bromide staining on the left. Absolute intensities were scored in arbitrary units, and the ratio between induced and uninduced signal is depicted for each gene.
mulation phenotype of the mutant F77A. Most striking was the loss of nucleocytoplasmic shuttling during nuclear accumulation (Fig. 2C), which was also demonstrated during treatment with the kinase inhibitor staurosporine (Fig. 2B). Importantly, the phenotype of F77A reveals new details about the role that DNA binding plays in the nuclear accumulation of Stat1. It was hypothesized that access to a nuclear export signal within the DNA binding domain was regulated by DNA binding (45). According to this model, DNA binding would mask the nuclear export signal and thus preclude nuclear export. Consequently, loss of DNA binding would inadvertently result in loss of nuclear accumulation. However, this is not the case. Nuclear retention of DNA binding mutants can be restored by treatment of cells with phosphatase inhibitors (7), or as is shown in Fig. 2D, by additional mutation of the interface of Stat1 that specifically controls its dephosphorylation. These results confirm the finding that tyrosine-phosphorylated Stat1 is retained in the nucleus (7), which makes dephosphorylation the crucial step in the control of nuclear export. For this reason it is dimerization, and not subsequent DNA binding, that masks the region required for Stat1 nuclear export. Whether this region is indeed localized in the DNA binding domain awaits further study. Thus, DNA binding is not necessary, but sufficient, for nuclear retention of Stat1, because the DNA bound molecule is refractory to phosphatase attack.

At the same time also the concomitant tetramerization defect of the mutant F77A influenced nuclear accumulation, which became apparent when we examined the intranuclear mobility during cytokine stimulation. Interestingly, statistically significant mobility differences between wild type and mutant Stat1 were found only during vanadate treatment. The addition of vanadate, which inhibits tyrosine phosphatase activity, results in increased nuclear concentration of Stat1 dimers. It is reasonable to assume that oligomerization of Stat1 is favored in this situation. However, due to its tetramerization defect the mutant will form oligomers less frequently and thus maintain a comparatively high mobility even in the face of its intrinsic dephosphorylation defect. Nevertheless, despite its high intranuclear mobility, a feature that discriminates this protein from Stat1-DNA plus, which also displays prolonged nuclear retention (7), the concurrent dephosphorylation defect of mutant F77A prevented its nuclear export. Thus, because loss of dephosphorylation was associated with diminished oligomerization, the mutant F77A displayed a unique phenotype where efficient nuclear trapping was combined with elevated intranuclear mobility.

Our transcription analyses have indicated that mutation of Phe\(^{77}\) influences gene induction in a promoter-specific manner. Transcription from transiently transfected “strong” synthetic promoters was elevated with the tetramerization-deficient Stat1 and Stat5, whereas reduced transcription was seen when natural promoter fragments were used in this kind of assay. These results clearly indicated that the mutant Stats are functional transcription factors that retained cytokine responsiveness. RT-PCR analysis of natural IFN\(\gamma\) or IFN\(\alpha\)-dependent target genes in their native chromatin setting demonstrated reduced transcriptional activity of the Stat1 mutant protein. At present the molecular defects underlying these phenotypes are not clear. Together, the reporter gene results with synthetic promoters argue for an influence of tetramerization deficiency, since optimal GAS sites are characterized by a markedly lower DNA off rate as compared with many physiological sites. Therefore effective binding to such sites is less dependent upon cooperativity (18). The multisimerization of optimal sites may mimic oligomerization, while the natural promoter fragments that were used contained “weak” sites and thus required Stat oligomerization for transcriptional activity. We note that the ICAM-1 enhancer not only contains a single canonical GAS site but additionally a non-canonical GAS site (5-TCCCGAGG) just 14 bp upstream.\(^3\) Somewhat surprisingly, also an IFN\(\gamma\)-responsive promoter with only a single discernable GAS site, such as IRF-1 (44), was susceptible to the mutation of Phe\(^{77}\), despite the fact that binding of the mutant F77A to single (not shown) or tandem IRF-1 sites (Fig. 1G) was indistinguishable from wild type. This may indicate that Stat tetramers are mandatory for full transcriptional activity at many promoters that contain only a single canonical GAS site. This interpretation is supported by the DNA binding data shown in Fig. 1, H and I. Clearly, the ability to form tetramers (or higher order polymers) on DNA did not require multiple GAS elements. Therefore, it is likely that tetramers of Stat1 are also bound at promoters that contain only a single canonical GAS site. This situation may not be limited to Stat1. In vitro binding site selection revealed that tetrameric binding of Stat5 also can be seen with a wide range of non-consensus motifs, which in many cases did not allow Stat5a binding as a dimer (28). Additionally, it was shown before that the N-domain is dispensable for high affinity DNA binding (18, 19), and Fig. 1, D and E, show that mutation of Phe\(^{77}\) or Phe\(^{81}\) in Stat1 or Stat5, respectively, did not diminish interactions of the dimers with DNA.

Although mutation of residue 77 of Stat1 or 81 of Stat5 was without adverse effects on gene induction from promoters containing multiple strong GAS elements, an alternative explanation for the observed reduced transcription from natural promoters must be considered, since the N-domain has a role also in the recruitment of transcriptional co-activators (45). At present we cannot exclude the possibility that the mutation of residue Phe\(^{77}\) modulates also recruitment of unrelated transcription factors. Further analysis is required to decide this point.

In summary, the interface centered around the critical phenyalanine fulfills distinct functions that are subject to control by the tyrosine phosphorylation and DNA binding status of the Stat molecule. The N-domains of unphosphorylated Stats appeared not to interact with the phosphatase (Fig. 3D). In the activated molecule, however, Phe\(^{77}\) participates in recruitment of the phosphatase (Fig. 3C), whereas during DNA binding the Stat1 protein is protected from dephosphorylation (7). At present no structural data with regard to the position of the N-domain in phosphorylated and unphosphorylated Stats are available. It is therefore not possible to describe the various states in structural terms. From this and other work (22, 36, 45) it becomes clear that the surface of the Stat N-domain influences a remarkable diversity of crucially important functions, and despite high structural conservation the degree of functional variability appears to be considerable.

Acknowledgments—We thank Paul T. van der Saag, Hubrecht Laboratory, Utrecht, and Marc Vigneron and Claude Kedinger, Université de Strasbourg, for valuable reagents.

REFERENCES

1. Darnell, J. E., Jr. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11767–11769.
2. Bale, J. N., and Kerr, I. M. (1995) Trends Genet. 11, 69–74.
3. Leaman, D. W., Leung, S. L. X., and Stark, G. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1578–1588.
4. Shuai, K., Stark, G. R., Kerr, I. M., and Darnell, J. E., Jr. (1993) Science 261, 1744–1776.
5. Shuai, K., Horvath, C. M., Huang, L. H., Qureshi, S. A., Cowburn, D., and Darnell, J. E. (1994) Cell 76, 821–826.
6. Greenland, A. C., Morales, M. O., Viviano, B. L., Yan, H., Krolevski, J., and Schreiber, R. D. (1995) Immunity 2, 677–687.
7. Meyer, T., Marg, A., Lemke, P., Wiesen, B., and Vinkemeier, U. (2003) Genes Dev. 17, 1992–2005.
8. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421.

\(^3\) U. Vinkemeier, unpublished observation.
A Single Residue Modulates Tyrosine Dephosphorylation, Oligomerization, and Nuclear Accumulation of Stat Transcription Factors
Thomas Meyer, Lisa Hendry, Andreas Begitt, Susan John and Uwe Vinkemeier

J. Biol. Chem. 2004, 279:18998-19007.
doi: 10.1074/jbc.M400766200 originally published online March 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400766200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 28 of which can be accessed free at http://www.jbc.org/content/279/18/18998.full.html#ref-list-1