

Amino-terminal Signal Transducer and Activator of Transcription (STAT) Domains Regulate Nuclear Translocation and STAT Deactivation*

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The first ~100 amino acids of the STAT (signal transducer and activator of transcription) family of transcription factors share a high degree of sequence similarity. To determine whether they encode a functionally conserved domain, amino-terminal chimeric STATs were created. These chimeric STATs share a number of properties with wild-type Stat1, including a predominantly cytoplasmic pattern of expression in unstimulated cells. Upon stimulation with ligand, the chimeric STATs rapidly become tyrosine-phosphorylated, dimerize, and are able to bind DNA. They are also able to heterodimerize with coexpressed wild-type Stat1. Yet in contrast to wild-type Stat1, the chimeric STATs exhibit a marked defect in deactivation. Moreover, the persistence of active chimeras correlates directly with an inability to translocate to the nucleus. The defects both in nuclear translocation and in deactivation are rescued by heterodimerization with coexpressed wild-type Stat1. This study indicates that STAT amino termini provide a signal that is important for nuclear translocation and, subsequently, deactivation. It also suggests that deactivation may depend on a prior nuclear localization event.

Cytokines mediate their pleiotropic effects on cells by binding to specific transmembrane-spanning receptors. These receptors transduce signals into the cell, culminating in the induction of new genes. Characterization of the ability of IFNs¹ to rapidly induce new genes has led to the elucidation of the JAK-STAT (signal transducer and activator of transcription) signaling pathway, which is now known to transduce signals for other cytokines as well (1, 2). JAKs are receptor-associated tyrosine kinases that mediate ligand-dependent receptor phosphorylation. Receptor phosphotyrosyl residues are, in turn, specifically recognized by the SH2 domain of members of the STAT family of cytoplasmic transcription factors (3–5). Once recruited to the receptor, STATs are activated by a single tyrosine phosphorylation event. Activated STATs are released from the receptor and then heterodimerize through the inter-

action between the phosphotyrosine of one STAT and the SH2 domain of another STAT (6–9). These active heterodimers translocate to the nucleus by a poorly understood mechanism and bind to a member of the GAS family of enhancers (1, 10–12).

Seven STATs, ranging in size from ~90 to ~115 kDa, have been reported in mammals (1, 2, 13). Sequence comparison of these STATs has led to the identification of several well conserved domains, the most highly conserved of which is the SH2 domain (7, 8). Carboxyl-terminal to this domain is the tyrosine that becomes activated in response to ligand and subsequently mediates dimerization (10, 14, 15). Amino-terminal to this domain, but separated by an ~80-amino acid linker region, is the DNA-binding domain (6, 9, 16). There are several additional conserved amino-terminal domains whose structures suggest they may mediate interactions with other proteins. Of these, the first ~100 amino acids are most conserved. Recent structural and functional studies suggest that this domain promotes cooperativity of DNA binding by mediating an interaction between two STAT dimers (6, 9, 17, 18). As expected, mutagenesis of an invariant tryptophan (Trp-37 for Stat1) that is critical for this interaction abrogates cooperativity in DNA binding (18). Mutation of another highly conserved residue in this interaction domain (Arg-31) has been shown to correlate with persistent tyrosine phosphorylation (19). Although the structure of this region does not support a role for Arg-31 in mediating an interaction with a phosphatase, as initially argued, these observations do suggest that the amino-terminal domain is likely to serve an important regulatory function(s) in addition to cooperativity of DNA binding.

To determine whether these highly conserved amino-terminal domains are functionally conserved and to evaluate the possibility that they may participate in other aspects of STAT signaling, chimeric STATs were generated. As anticipated, when the amino terminus of Stat1 (amino acids 1–129) was replaced with the homologous regions of Stat2 or Stat5, many functional properties were conserved. However, these chimeras exhibited a marked defect in nuclear translocation and deactivation.

EXPERIMENTAL PROCEDURES

Plasmids—Chimeric receptors were generated by introducing DNA encoding amino acids 437–518 from the endodomain and transmembrane domain of the human IFN- α receptor α -chain (20) into R_cCMV (Invitrogen) encoding the 2200-base pair ectodomain of the human G-CSF receptor (21). Oligonucleotides encoding the Stat1-binding/recruitment site (TSFGYDKPHV) from the human interferon- γ receptor α -chain (5) and a triple Myc tag (3) were added to the 3'-end of the receptor chimera to yield the mature construct G/aR α . The CH2/1 construct was generated by introducing a polymerase chain reaction product encoding amino acids 1–140 of Stat2, through blunt end cloning, into an R_cCMV-based expression construct encoding amino acids 130–750 of Stat1. Similarly, the STAT CH5/1 construct was prepared

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¹ The abbreviations used are: IFNs, interferons; JAK, Janus kinase; GAS, interferon- γ activation sequence; G-CSF, granulocyte colony-stimulating factor; EMSA, electrophoretic mobility shift assay; WCE, whole cell extract; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription.

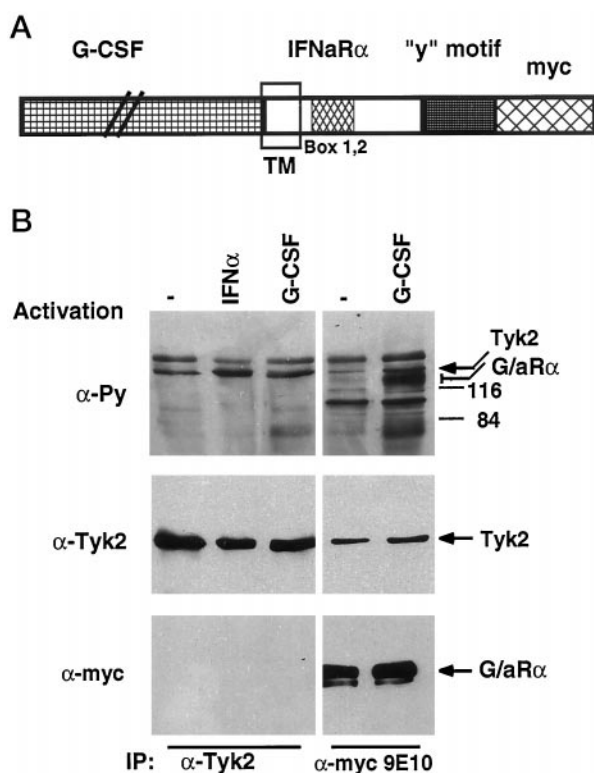


FIG. 1. Artificial chimeric receptor G/aR α is responsive to G-CSF in 293 cells. *A*, structure of the chimeric G/aR α receptor. This receptor consists of the extracellular domain of the human G-CSF receptor (21) fused to the transmembrane (TM) and proximal intracellular domains of the IFN- α receptor α -chain (IFN α R α ; IFNAR1), which mediates the interaction with and activation of Tyk2 (20). Juxtaposed to the 3'-end of the JAK-binding motif is the Stat1 recruitment, or tyrosine motif ("y" motif), from the human IFN- γ receptor α -chain (5) and a triple Myc epitope tag (3). *B*, functional analysis of G/aR α expressed in 293 cells. 293 cells were transfected with G/aR α . These cells were either left untreated or stimulated with G-CSF or IFN- α for 15 min as indicated. Immunoprecipitates (IP) of WCEs (collected by an anti-Myc epitope or anti-Tyk2 antibody) were then fractionated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblots were performed with an anti-phosphotyrosine (α -Py; 4G10), anti-Tyk2, or anti-Myc epitope antibody.

by the introduction of a polymerase chain reaction product encoding amino acids 1–129 of Stat5b into the same Stat1 expression construct (*i.e.* amino acids 130–750). Each of these constructs was confirmed by sequencing. The generation of Δ NStat1 (amino acids 135–750) has been previously described (22).

Cell Culture and Transfections—The human kidney fibroblast cell line 293 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). For transfection, 293 cells were pretreated for 5 min with 25 mM chloroquine (Sigma) and then transfected with 10 μ g of the STAT constructs and 10 μ g of G/aR α by the calcium phosphate method (23). After 24 h, transfected cells were reseeded and, after another 24 h, stimulated with human recombinant G-CSF (250 ng/ml; Amgen) or IFN- α (1000 units/ml; Genentech). As indicated, in some studies, staurosporine (dissolved in Me₂SO; Sigma) was added to a final concentration of 500 nM.

DNA Binding Assays and Cell Extracts—Electrophoretic mobility shift assays (EMSA) were carried out with an IRF1 GAS probe (gatcGATTTCCCGAAAT; Oligos Etc.) as described previously (24–26). Cytoplasmic, nuclear, or whole cell extracts (WCEs) were made as reported previously (10, 25, 27). For supershifts, 0.6 μ l of anti-STAT antibodies were added to a standard DNA binding reaction (12.5 μ l for 20 min at 25 °C) and incubated at 4 °C for an additional hour prior to EMSA. In some studies, cytoplasmic extracts were treated with calf intestinal phosphatase (Buffer 2, New England Biolabs Inc.) for 1 h at 37 °C prior to evaluation by EMSA.

Immunoblotting Assays and Antibody—Immunoprecipitations and immunoblotting were performed as described previously (28). Briefly, immunoprecipitates were fractionated by 7% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immu-

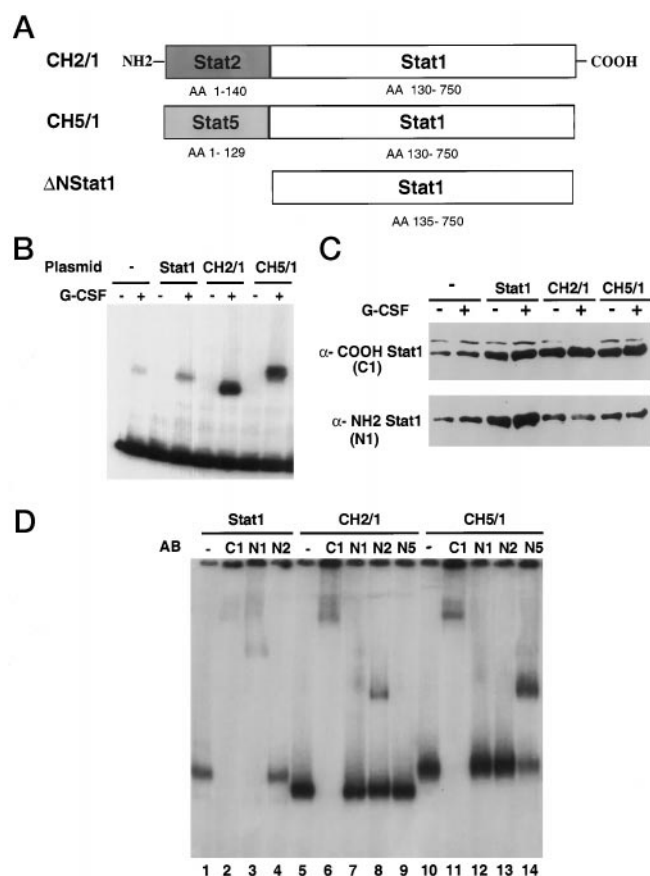


FIG. 2. Characterization of activated chimeric STATs. *A*, structure of mutant STAT constructs. The amino terminus of Stat1 (amino acids (AA) 1–129) was replaced by either amino acids 1–140 of Stat2 or amino acids 1–129 of Stat5. Δ NStat1 is missing amino acids 1–134. *B*, mobility shift assay of chimeric STATs. WCEs prepared from unstimulated or G-CSF-stimulated 293 cells transfected with G/aR α and/or STAT constructs were evaluated by EMSA with an IRF1 GAS probe. *C*, chimeric STATs and wild-type Stat1 are expressed at equivalent levels in transfected cells. WCEs from *B* were fractionated by SDS-polyacrylamide gel electrophoresis and sequentially immunoblotted with Stat1-specific antibodies C1 (carboxyl terminus) and N1 (amino terminus). *D*, supershift of wild-type and chimeric STAT DNA-binding complexes. WCEs from *B* were incubated with the indicated STAT-specific antibodies (AB) and then evaluated by EMSA as described for *B*. C1 recognizes the carboxyl terminus of Stat1 (lanes 2, 6, and 11); N1 recognizes the amino terminus of Stat1 (lanes 3, 7, and 12); N2 recognizes the amino terminus of Stat2 (lanes 4, 8, and 13); and N5 recognizes the amino terminus of Stat5 (lanes 9 and 14).

noblotted with the following: a phosphotyrosine-specific antibody (4G10; Upstate Biotechnology, Inc.), a Myc epitope-specific antibody (9E10; Santa Cruz Biotechnology), a Tyk2-specific antibody (Santa Cruz Biotechnology), or STAT-specific antibodies. STAT-specific antibodies included those recognizing the amino terminus of Stat1 (N1 (29)), the carboxyl terminus of Stat1 (C1 (29)), the amino terminus of Stat2 (monoclonal N2; Transduction Laboratories); and the amino terminus of Stat5 (N5, prepared against a glutathione S-transferase fusion protein of Stat5b amino acids 1–49 (28)). N5 represents an IgG fraction prepared from crude serum (Pierce). Immunoblotted bands were detected by ECL as specified by the manufacturer (Amersham Pharmacia Biotech). For sequential Western blots, filters were stripped in 2% SDS, 65 mM Tris (pH 6.7), and 100 mM β -mercaptoethanol for 45 min at 50 °C.

Immunofluorescence Studies—Cells were rinsed twice in phosphate-buffered saline (PBS) and fixed in 2% formaldehyde/PBS. After two additional PBS rinses, cells were treated with 1% Triton X-100 (in PBS; Sigma) for 2 min and then washed in 0.1% Tween 20 (in PBS; Sigma). Nonspecific protein adsorption was blocked by a 30-min incubation with 2% bovine serum albumin/PBS. Samples were incubated with primary antibody (C1; 1:200 in 2% bovine serum albumin/PBS) for 1 h (22 °C), washed, and then incubated with secondary antibody (Cy3-conjugated donkey anti-rabbit antibody, 1:300; Jackson ImmunoResearch Labora-

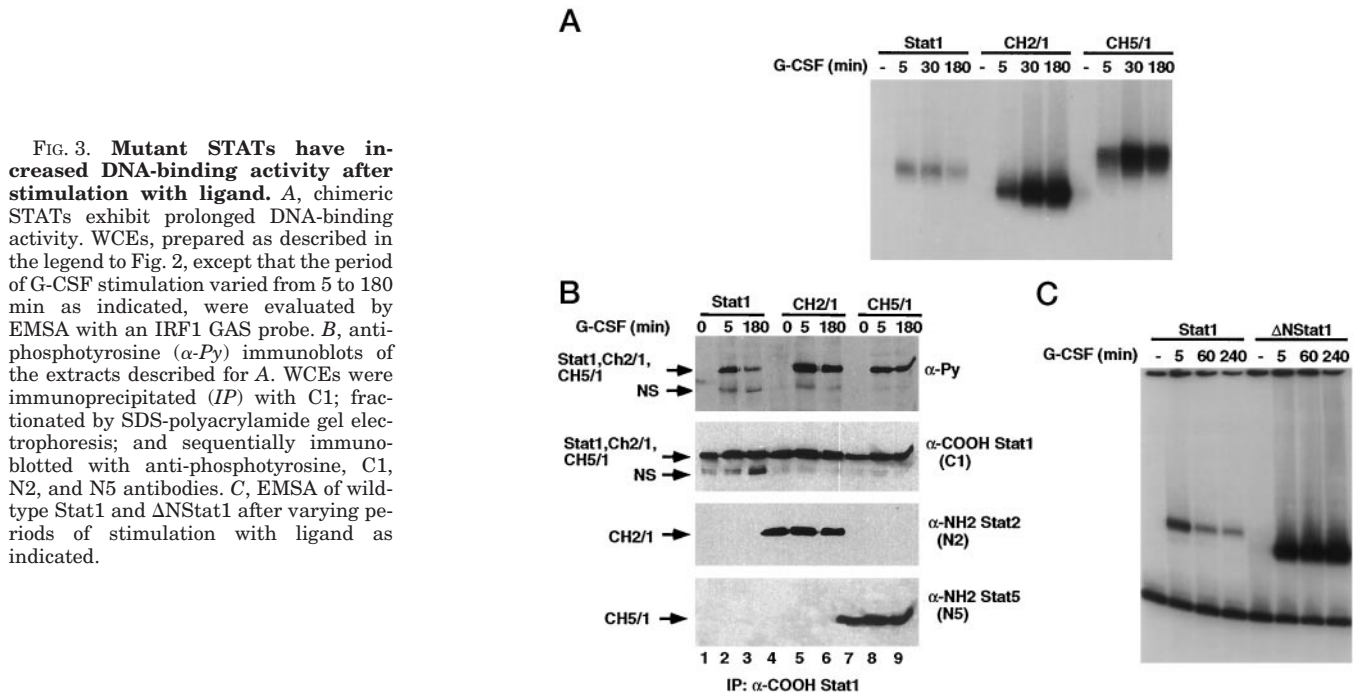


FIG. 3. Mutant STATs have increased DNA-binding activity after stimulation with ligand. *A*, chimeric STATs exhibit prolonged DNA-binding activity. WCEs, prepared as described in the legend to Fig. 2, except that the period of G-CSF stimulation varied from 5 to 180 min as indicated, were evaluated by EMSA with an IRF1 GAS probe. *B*, anti-phosphotyrosine (α -Py) immunoblots of the extracts described for *A*. WCEs were immunoprecipitated (IP) with C1; fractionated by SDS-polyacrylamide gel electrophoresis; and sequentially immunoblotted with anti-phosphotyrosine, C1, N2, and N5 antibodies. *C*, EMSA of wild-type Stat1 and Δ NStat1 after varying periods of stimulation with ligand as indicated.

ories, Inc.) for 30 min. These samples were washed and examined at a magnification of $\times 400$ under a Zeiss LSM410 laser scanning confocal microscope.

RESULTS

Development of a Rapid Assay to Evaluate Chimeric STATs—293 cells were selected to evaluate the function of mutant STATs because of their high transfection efficiency. However, as recent studies suggested that the amino-terminal domains of STATs may be required for a productive interaction with an appropriate receptor (30), we set out to design a generic receptor that would activate wild-type and amino-terminal chimeric STATs with equivalent efficiency. The extracellular domain of the G-CSF receptor was selected as the ligand-binding domain because only one type of receptor subunit is required for ligand binding and because of its limited pattern of tissue expression (*e.g.* not in 293 cells (21)). The endodomain included the minimal region of the IFN- α receptor α -chain (IFNAR1) implicated in JAK (*i.e.* Tyk2) binding/activation (20, 31) and the 10-amino acid Stat1 recruitment/activation motif from the IFN- γ receptor α -chain (5). This chimeric receptor is referred to as G/aR α .

To test the G/aR α receptor functionally, it was introduced into 293 cells by transient transfection and then stimulated with G-CSF. When the chimeric receptor was collected from transfected cells by virtue of its carboxyl-terminal Myc epitope tag (3), it was found to be tyrosine-phosphorylated in response to ligand (Fig. 1B). Moreover, activated Tyk2 co-immunoprecipitated with the receptor. Activation of Tyk2 was also evaluated directly. Consistent with previous studies (20, 32), a basal level of phosphorylated Tyk2 was observed in unstimulated cells, which then increased upon stimulation with G-CSF (Fig. 1B). Stimulation of transfected cells with IFN- α , which activates the endogenous type I receptor, yielded a similar level of Tyk2 phosphorylation. These results demonstrate that the chimeric receptor and associated Tyk2 are activated in response to G-CSF in 293 cells. As anticipated, this receptor promotes the G-CSF-dependent activation of either endogenous or cotransfected Stat1 (see below). Experiments with control receptors demonstrated that the Stat1 recruitment tyrosine motif is required for STAT activation (data not shown). These results

indicate that this chimeric receptor will be a valuable tool for the activation of transfected Stat1 chimeras.

Generation of Chimeric STATs—To test whether the conserved ~ 100 amino acids at the amino terminus of STATs mediate a general or perhaps a more STAT-specific function(s), chimeric STAT molecules were prepared. To generate these chimeras, the amino terminus of Stat1 was carefully replaced by the homologous regions of Stat2 or Stat5 (Fig. 2A). Amino acid 130 was selected for the site of fusion because it is poorly conserved and appears to form a random coil (6, 9, 17, 18). We predicted that if the function(s) encoded by this domain were conserved, then the chimeric molecules would behave similarly to wild-type Stat1. But, if they encoded STAT-specific functions, then these molecules should be functionally crippled.

First, we determined whether these chimeric STATs were activated by the G/aR α receptor. Extracts were prepared from 293 cells cotransfected with both constructs, either before or after stimulation with G-CSF, and then evaluated by EMSA (Fig. 2B). 293 cells transfected with G/aR α alone exhibited activation of endogenous Stat1 α . The level of Stat1 DNA-binding activity increased when Stat1 was cotransfected. Surprisingly, cells cotransfected with the 2/1 or 5/1 chimeras (*i.e.* CH2/1 and CH5/1) demonstrated a more robust DNA-binding activity than wild-type Stat1 (either endogenous or recombinant). Moreover, the chimeric STATs exhibited a distinct mobility. Immunoblotting these extracts with a Stat1 carboxyl terminus-specific antibody (C1; an epitope that is present in each STAT construct) confirmed that each of these proteins was overexpressed to a similar level (Fig. 2C).

To further characterize these DNA-binding complexes, they were probed with antibodies directed either against the conserved Stat1 carboxyl terminus (C1) or their unique amino termini (N1 for Stat1, N2 for Stat2, and N5 for Stat5 (Fig. 2D)). Each of the chimeric complexes was recognized (*i.e.* supershifted or blocked) by C1. As anticipated, only the 5/1 chimera (CH5/1) was recognized by N5. Similarly, only the 2/1 chimera (CH2/1) interacted, albeit modestly, with N2. (N2 is a monoclonal antibody and not very effective at supershifting.) These studies demonstrate that chimeric STATs, activated through stimulation of a cotransfected receptor, each give rise to a novel

DNA-binding complex. Moreover, although these STATs are all expressed at equivalent levels, the chimeric STATs give rise to more intense shift bands.

Kinetics of Activation of Chimeric STATs Differ from Those of Stat1—The initial characterization of chimeric STAT DNA-binding activity was done 30 min after stimulation. To determine whether the observed differences in DNA-binding activity were dependent on the duration of stimulation, a kinetic study was undertaken. Transfected cells were stimulated with G-CSF for 5, 30, and 180 min. Consistent with published studies (33, 34), wild-type Stat1 was maximally phosphorylated by 5 min. Densitometric evaluation indicated that there was a 2.5–3-fold loss of DNA-binding activity by 180 min (Fig. 3A). Evaluation of the chimeric STATs revealed some notable differences. Early after stimulation, chimeric STATs were phosphorylated to similar level as Stat1. However, their level of phosphorylation continued to increase at 30 min and exhibited little decay at 180 min after stimulation. Analogous results were obtained when phosphorylation was evaluated by a less sensitive immunoblotting assay (Fig. 3B). As anticipated, the immunoblotting assay found a decrease in the level of Stat1 phosphorylation after 180 min of stimulation. In contrast, the chimeric STATs remained heavily phosphorylated at this time point. However, this assay failed to demonstrate an increase in chimeric STAT phosphorylation at 180 min. Rather, there was a modest decrease. This suggests that changes other than those in the level of tyrosine phosphorylation (*e.g.* structural; see below) may contribute to the increase in DNA-binding activity. A faster migrating band (Fig. 3B, *left panel*, labeled *NS*) represents a very low abundance, amino-terminally truncated isoform of Stat1 that can be detected only after substantial enrichment by immunoprecipitation from 293 cell extracts (data not shown). The filter was then reprobbed with STAT-specific antibodies C1, N2, and N5 to demonstrate that the transfected STATs were immunoprecipitated to the same extent. These results demonstrate equivalent levels of phosphorylation at early times and suggest that wild-type and chimeric STATs are equipotent substrates for the JAK kinases. The relative increase in the amount of activated (*i.e.* phosphorylated) chimeric STAT is consistent with a process of accumulation (*i.e.* a defect in deactivation; see below).

To determine whether the apparent defect in deactivation was due to either the presence of new amino-terminal sequences (*i.e.* Stat2 or Stat5) or the loss of Stat1 amino-terminal sequences, an amino-terminal Stat1 deletion mutant (*i.e.* Δ NStat1 (22)) was examined. Evaluation of the kinetics of G-CSF-stimulated Δ NStat1 demonstrated a prolonged pattern of activation (*i.e.* DNA binding), analogous to what was observed for the chimeric STATs (Fig. 3C). However, the level of Δ NStat1 activation was even higher and more rapid than observed for the chimeric STATs. Immunoblotting studies confirmed that Δ NStat1 was expressed at levels similar to wild-type and chimeric Stat1 (data not shown). These observations indicate that a loss of Stat1 amino-terminal sequences leads to an increase in the level of phosphorylation. The domain(s) encoded in the amino terminus of Stat2 or Stat5 do not compensate for this loss.

Level of Chimeric STAT Phosphorylation Is Not Sensitive to Staurosporine—Recent studies demonstrating that the duration of ligand-stimulated Stat1 DNA-binding activity is sensitive to kinase inhibitors indicated that prolonged DNA binding is dependent on continuous JAK activity (33). Since chimeric STATs bind DNA long after JAK activity is likely to have decayed, their prolonged biological activity appears to be independent of kinase activity. To test this, the kinetics of wild-type and chimeric Stat1 DNA-binding activities were evaluated in

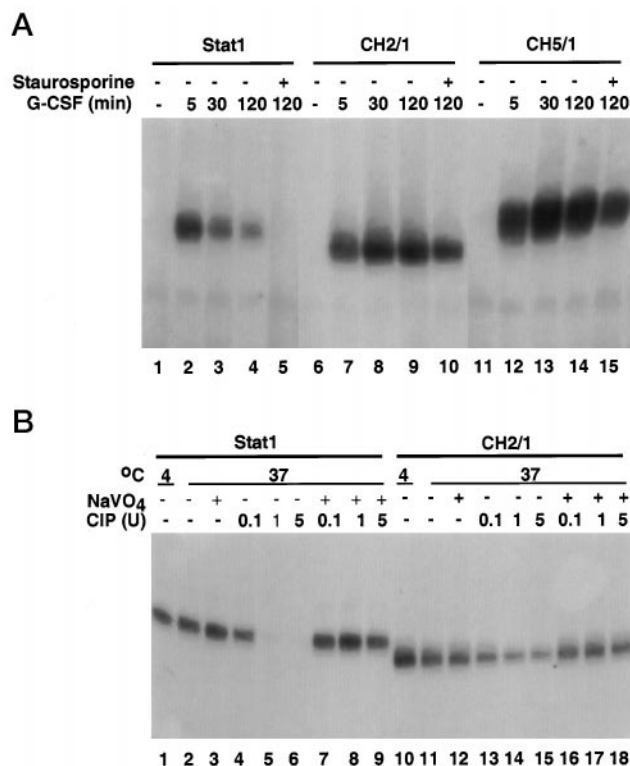


FIG. 4. Activated chimeric STATs have a decreased sensitivity to staurosporine and phosphatase treatment. *A*, staurosporine sensitivity of wild-type and chimeric STATs. WCEs were prepared from transfected 293 cells as described in the legend to Fig. 2, except that 1 h after stimulation, some were treated with 500 nM staurosporine for 60 min. Cells were stimulated with G-CSF for varying amounts of time as indicated and then evaluated by EMSA with an IRF1 GAS probe. *B*, calf intestinal phosphatase (*CIP*) sensitivity of wild-type and chimeric STATs. WCEs prepared as described in the legend to Fig. 2 were incubated with 0.1–5 units (*U*) of calf intestinal phosphatase (20 min at 37 °C) as indicated and then evaluated by EMSA with an IRF1 GAS probe. Sodium orthovanadate (1 mM) was used to block phosphatase activity in some samples (*lanes 7–9 and 16–18*). Controls included samples incubated at 4 °C in the absence of phosphatase (*lanes 1 and 10*).

the presence and absence of staurosporine, a JAK inhibitor (Fig. 4A). Consistent with published studies, the DNA-binding activity of wild-type Stat1 was lost after 1 h of staurosporine treatment (Fig. 4A, *lane 5*). In contrast, the DNA-binding activity of the chimeric STATs was only modestly affected by the addition of staurosporine (Fig. 4A, *lanes 10 and 15*), indicating that only a fraction of the prolonged DNA-binding activity can be attributed to persistent kinase activity. Rather, these observations imply that an important cause for the increased amount of phosphorylated chimeric STATs is also due to a defect in deactivation.

STAT Sensitivity to a Nonspecific Phosphatase—The preceding studies indicated that chimeric STATs accumulate in a phosphorylated form due to a defect in deactivation. However, they failed to provide any insight into whether this can be attributed to a structural change and/or a failure to localize to an appropriate compartment (*e.g.* where phosphatases may be active). Recently published studies suggested that a conserved amino-terminal STAT domain may promote interactions with a phosphatase (19), but the crystal structure does not support this notion (18). Moreover, a swap between two conserved domains would not be expected to affect a general function like the ability of STATs to interact with a phosphatase. Evaluation of the mobility of the chimeric STAT-DNA complexes indicated they differed from that of wild-type Stat1 (Figs. 2–4). This

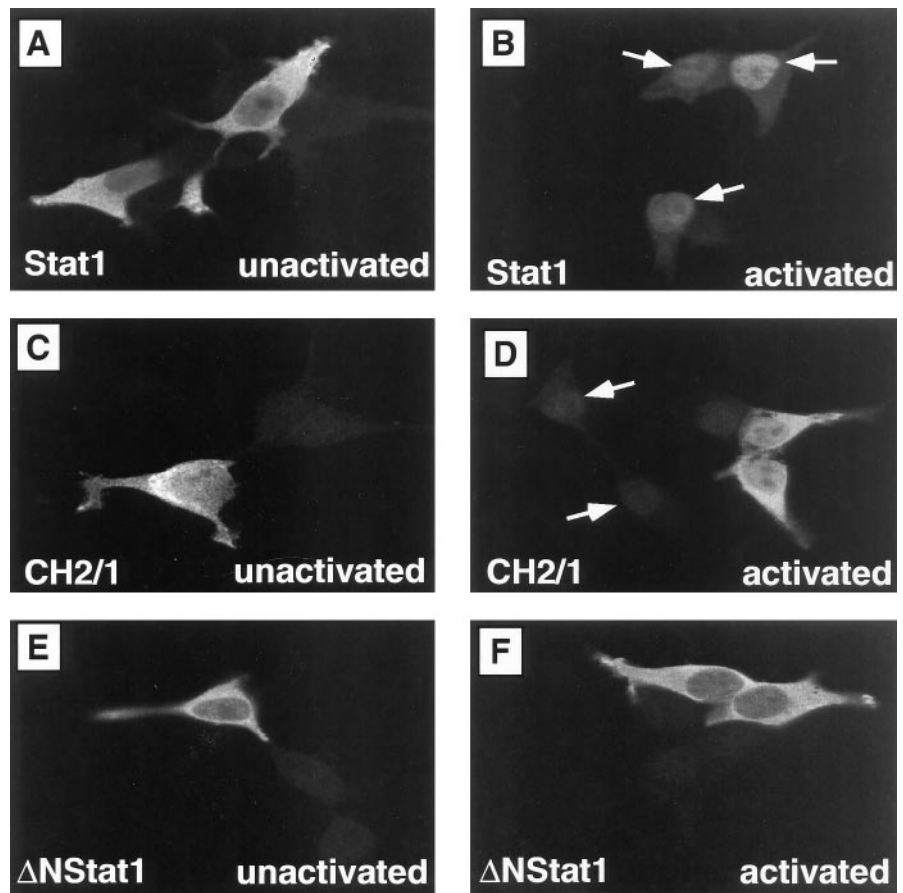


FIG. 5. Immunofluorescence studies of 293 cells transfected with wild-type or mutant STATs. Wild-type Stat1, CH2/1, or Δ NStat1 was transfected into 293 cells and stained with the Stat1 carboxyl terminus-specific antibody C1 either before (A, C, and E) or after (B, D, and F) a 30-min stimulation with 1000 units/ml IFN- α . Nuclear translocation of endogenous Stat1 is indicated by arrows in D. Primary antibody staining was visualized with a Cy3-conjugated secondary antibody under a Zeiss confocal microscope.

suggested that the chimeras have undergone a structural change, at least when bound to DNA. Yet, most STAT functions were unaffected (*e.g.* the ability to productively interact with the receptor-kinase complex, to dimerize, or to bind DNA), indicating that the structural change was subtle. To crudely probe the structural properties of wild-type and chimeric Stat1, they were treated with three doses of a potent nonspecific phosphatase (Fig. 4B). The DNA-binding activity of wild-type Stat1 was completely abrogated by 1 unit of phosphatase. In contrast, the 2/1 chimera had only a limited susceptibility to this phosphatase. A residual amount of DNA-binding activity was evident even after exposure to high doses of phosphatase. As anticipated, phosphatase activity was sensitive to sodium orthovanadate. Although these observations do not address the ability of chimeric STATs to potentially interact with native phosphatases, they do suggest that chimeric STATs exhibit a structural change that affects their ability to be nonspecifically dephosphorylated *in vitro*.

Amino-terminal Stat1 Domains Are Required for Nuclear Translocation—Next, we examined the possibility that the accumulation of phosphorylated chimeric STATs might be secondary to failure to localize to an appropriate cellular compartment. Since previous studies had suggested that STATs may be dephosphorylated in the nucleus (35), we considered a defect in nuclear translocation first. A sensitive immunofluorescence assay was selected to evaluate the ability of wild-type and chimeric STATs to translocate to the nucleus (10). In an effort to have the nuclear translocation of endogenous Stat1 (*i.e.* in nontransfected cells) serve as a positive control, we determined whether chimeric STATs could be activated through stimulation of an endogenous receptor. Comparison of DNA-binding activities confirmed that transfected STATs were activated similarly in response to stimulation of either endogenous IFN- α receptors or the cotransfected G/aR α receptor (data not

shown). As anticipated, in unstimulated transfected cells, both wild-type and chimeric STATs resided predominantly in the cytoplasm (Fig. 5, A and B). Similar observations were made for endogenous Stat1. When cells were stimulated with IFN- α , both endogenous (*e.g.* Fig. 5D) and transfected (Fig. 5B) Stat1 translocated to the nucleus as expected (10). In contrast, CH2/1 remained largely in the cytoplasm (Fig. 5D). Cells cotransfected with G/aR α and stimulated with G-CSF yielded equivalent results (Fig. 6). Consistent with our previous observations suggesting a functional similarity between Δ NStat1 and chimeric STATs (Fig. 3C), Δ NStat1 also exhibited a defect in ligand-dependent nuclear translocation (Fig. 5, compare E and F). Additional studies, up to 5 h after stimulation, confirmed a persistent defect in nuclear translocation (data not shown).

These compelling immunofluorescence results contrasted with those obtained through a more traditional cell fractionation approach, where some chimeric STATs were always recovered in the nuclear fraction (data not shown). Similarly, it was possible to demonstrate that chimeric STATs support a modest level of ligand-dependent activation of a cotransfected GAS-driven reporter gene (data not shown). Although these observations, along with the immunofluorescence studies, were consistent with a low level of ligand-dependent chimeric STAT nuclear translocation, we were puzzled as to how this might occur. One possibility was that the defect in chimeric STAT nuclear translocation was partially rescued through heterodimerization with the limiting amounts of activated endogenous Stat1. To test this, 293 cells were transfected with CH2/1 in the presence or absence of Stat1 β (*i.e.* Stat1/p84), a carboxyl-terminally truncated isoform of Stat1. Stat1 β was selected because it is not recognized by the Stat1 carboxyl terminus-specific antibody (C1), yet dimerizes with other STATs (*e.g.* Stat1, Stat2, and chimeric STATs (see Fig. 8 and Ref. 10)), translocates to the nucleus, and binds DNA normally (*e.g.* see

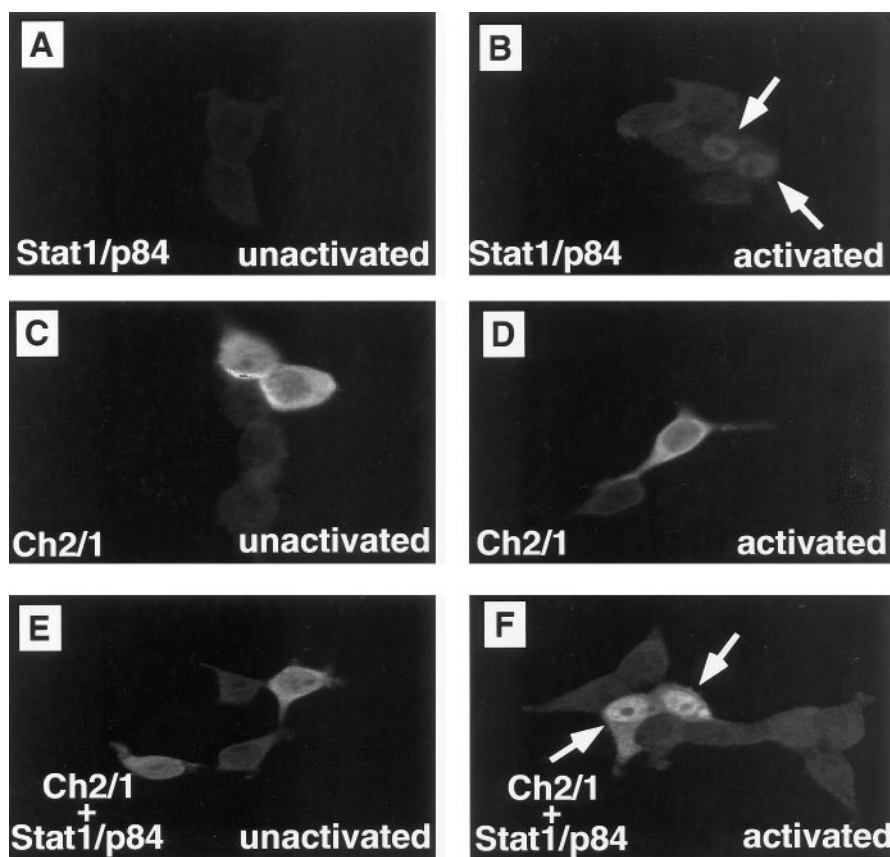


FIG. 6. Cotransfection of Stat1 β rescues the defect in CH2/1 nuclear translocation. Wild-type Stat1 β (*i.e.* Stat1/p84) and CH2/1 were transfected either independently (*A–D*) or together (*E* and *F*) into 293 cells. All cells were also cotransfected with G/aR α and immunostained either before (*A*, *C*, and *E*) or after (*B*, *D*, and *F*) stimulation with G-CSF as described in the legend to Fig. 5. Nuclear translocation is indicated with arrows in *B* and *F*. The C1 antibody recognizes endogenous Stat1 (*e.g.* *B*), but not cotransfected Stat1 β .

below). Therefore, only wild-type or chimeric STATs will be detected in cells cotransfected with Stat1 β (*e.g.* Fig. 6). Again, endogenous Stat1 translocated to the nucleus normally in response to ligand (Fig. 6*B*), but CH2/1 did not (Fig. 6, compare *C* and *D*). Upon cotransfection of Stat1 β , there was a dramatic increase in the amount of CH2/1 translocating to the nucleus (Fig. 6, compare *E* and *F*). Moreover, increasing the amount of cotransfected Stat1 β led to a dose-dependent increase in ligand-stimulated CH2/1 nuclear translocation (Fig. 7, *A* and *B*). In stark contrast, however, cotransfection of Stat1 β was not able to rescue the defect in Δ NStat1 nuclear translocation (Fig. 7*C*). This can probably be attributed to the inability of Δ NStat1 to heterodimerize with Stat1 β (data not shown). These studies indicate that coexpression of Stat1 β is able to rescue the defect in CH2/1 and CH5/1 nuclear translocation (data not shown).

Defect in Nuclear Translocation Correlates with the Defect in Deactivation—Our observations that STATs, defective in nuclear translocation, fail to become deactivated suggested the possibility that these two events may be causally linked. To test whether deactivation is dependent on the ability to translocate to the nucleus, we took advantage of the ability of cotransfected Stat1 β to rescue the defect in CH2/1 nuclear translocation. 293 cells were transfected either with CH2/1 alone or with CH2/1 plus Stat1 β and then stimulated with G-CSF. Extracts prepared from these and control cells were evaluated by EMSA on a 6% gel to optimize resolution of the “mixed complexes.” Control cells, which included those transfected with Stat1 β or with Stat1 plus Stat1 β , gave rise to three transient DNA-binding complexes (Fig. 8, lanes 1–6). These complexes have previously been identified as Stat1 β homodimers (the fastest complex), Stat1 α homodimers (the slowest complex), and Stat1 α -Stat1 β heterodimers (the in-between complex).² Consistent with the relatively low levels of endogenous Stat1 (*i.e.* Stat1 α), cells

transfected with Stat1 β exhibited only a modest amount of Stat1 α -Stat1 α DNA-binding activity. In both of the wild-type control transfections (*i.e.* Fig. 8, lanes 1–3 and 4–6), a Bio-Rad phosphorimager indicated a 4-fold decrease in DNA-binding activity for each complex over the 180-min time course. As expected, however, the “novel” DNA-binding complex in cells transfected with CH2/1 failed to decay (Fig. 8, lanes 10 and 11). (Note that the DNA complex representing endogenous Stat1 was relatively weak and essentially comigrated with the CH2/1 homodimer.) When the CH2/1 chimera was cotransfected with Stat1 β , the two prominent complexes (Stat1 β -CH2/1 and CH2/1-CH2/1) and a weak Stat1 β -Stat1 β complex were evident. The DNA binding kinetics of these two more prominent complexes were quite distinct. Although the DNA-binding activity of the CH2/1 homodimer was stable over the 180-min assay period (as expected), the activity of the Stat1 β -CH2/1 heterodimer decayed by 2.5–3-fold, indicating that the defect in deactivation had been rescued (Fig. 8, lanes 5 and 6). Similar studies with the Δ NStat1 mutant were uninformative because this mutant can not effectively heterodimerize with Stat1 β (data not shown). These observations provide strong support for our model in which nuclear translocation and STAT deactivation are causally linked.

DISCUSSION

STATs are a family of transcription factors that transduce vital signals for cytokines. They share a number of conserved domains, which mediate important signaling functions. The most highly conserved of these, the SH2 domain, mediates several critical steps in the JAK-STAT signaling cascade (1, 7). The first entails recruitment to the appropriate activated receptor complex (3, 5, 36). The second entails an obligate interaction with a phosphotyrosyl residue with the activating JAK kinase (7, 37). The third entails STAT dimerization, where the SH2 domain of one STAT binds the phosphotyrosyl residue of

² I. Strehlow, C. Park, and C. Schindler, unpublished observation.

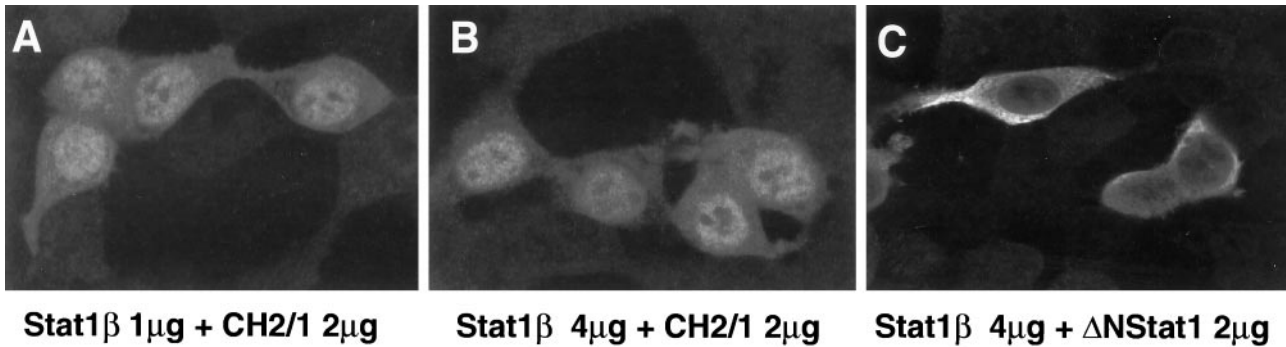


FIG. 7. Cotransfection of Stat1 β does not rescue the defect in Δ NStat1 nuclear translocation. 293 cells were transfected with Stat1 β and CH2/1 or Δ NStat1 and immunostained with the C1 antibody after stimulation of cotransfected G/aR α with G-CSF. In A and B, cells were transfected with a fixed amount of CH2/1 and an increasing amount of Stat1 β expression construct as indicated. In C, cells were transfected with 2 μ g of Δ NStat1 and 4 μ g of Stat1 β .

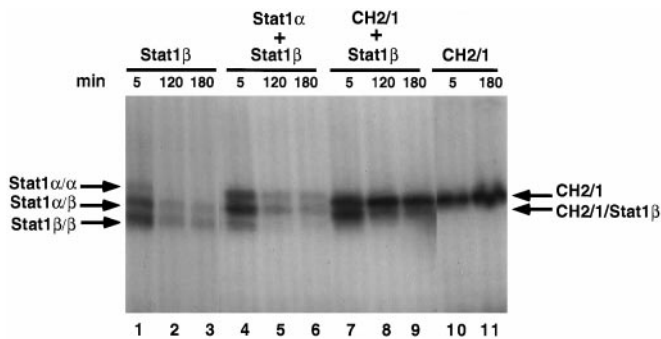


FIG. 8. Cotransfection of Stat1 β promotes deactivation of CH2/1. WCEs were prepared from 293 cells transfected with Stat1 α (*i.e.* the 91-kDa isoform), Stat1 β , and/or CH2/1 after varying periods of stimulation with G-CSF as indicated. They were then evaluated by EMSA with an IRF1 GAS probe on a 6% polyacrylamide gel. Cells were transfected with 2 μ g of CH2/1 or Stat1 α and 10 μ g of Stat1 β .

another STAT (5, 7, 8). This dimerization appears to be crucial for all subsequent steps in the signaling cascade, including the poorly understood process of nuclear translocation and, subsequently, DNA binding (6, 9, 10, 16, 34).

This phosphotyrosyl-dependent STAT signaling cascade is also counter-regulated at several steps. First, in unstimulated cells, STATs reside in the cytoplasm in an inactive conformation (10, 34). Upon stimulation, downstream regulatory molecules, including SOCS (suppressors of cytokine signaling (38)) and tyrosine phosphatases, are recruited to the activated receptors along with STATs (3, 39, 40)). SOCS, which encode an SH2 domain, appear to antagonize signaling by competing for binding to important phosphotyrosyl residues (38, 41, 42). In contrast, the phosphatases inactivate signaling molecules directly. There is compelling evidence that cytokine receptors and JAKs are actively dephosphorylated (33, 39, 40, 43). However, data on STAT deactivation remain more controversial. One recent study has suggested that STATs are deactivated through proteasome-mediated degradation, presumably after nuclear translocation (44). Other studies do not support this conclusion and indicate that STATs have a relatively long half-life and are deactivated by dephosphorylation (33, 45). Our studies support the later model because they find no changes in the level of STATs before and after stimulation. They also suggest that STATs are not deactivated until after they have translocated into the nucleus (see below).

Recent crystal structures of a small amino-terminal and large carboxyl-terminal STAT fragment have confirmed an important structural role for several conserved domains (6, 9, 18). The amino-terminal domain crystallized as a dimer, supporting its role in mediating interactions between STAT dimers bound

to adjacent GAS elements. However, both structures indicate that the two amino termini within one STAT dimer will not be able to interact. This suggests that they are free to promote other interactions. Consistent with this possibility, mutation of Arg-37, which plays a critical role in stabilizing an amino-terminal ring structure, leads to a defect in STAT deactivation (19). Although it had initially been argued that these data indicated that Arg-37 mediates an obligatory interaction with a phosphatase, the crystal structure demonstrates that this residue is not exposed. Rather, it predicts that a mutation of Arg-37 will destabilize the amino-terminal ring structure.

To more carefully study the function of the conserved \sim 100 amino acids at the STAT amino terminus, amino-terminal chimeric Stat2/Stat1 and Stat5/Stat1 proteins were generated. It was anticipated that if the functions encoded by this domain were conserved, then the chimeric molecules would behave like wild-type Stat1. But if they were STAT-specific, then these molecules would behave abnormally. When the chimeric molecules were examined, they retained many normal functional properties. These included activation by a receptor, dimerization, and DNA binding. However, they also exhibited a number of abnormal properties, including a unique mobility on EMSA, a defect in deactivation, and a defect in nuclear translocation. These defects are unlikely to be due to gross structural changes for several reasons. 1) The amino-proximal domain is structurally independent from the DNA-binding/dimerization core (6, 9). 2) Most of the functional properties of these molecules are conserved (see above). 3) The defect in nuclear translocation and deactivation can be rescued through heterodimerization with wild-type Stat1. Nonetheless, subtle structural changes in the chimeric STATs, manifested by an alteration in mobility on a native gel (Fig. 2B) and phosphatase sensitivity (Fig. 4A), suggest that the amino termini contribute to STAT structure. Moreover, an apparent increase in the relative DNA-binding affinity of chimeric STATs (Fig. 4) also supports a role for amino termini in STAT structure.

The correlation between a defect in nuclear translocation and deactivation suggests that these two processes are causally linked. However, the magnitude in the persistence of chimeric STAT DNA-binding activity may be multifactorial. For example, the studies in Fig. 3 suggest that chimeric STATs may exhibit an inherently better DNA-binding activity. Additionally, a defect in nuclear translocation may enhance tyrosine phosphorylation of chimeric STATs through an increased period of exposure to the activating kinase. Although the ability of coexpressed wild-type Stat1 to rescue the defect in both nuclear translocation and deactivation indicates that nuclear translocation is important for the deactivation process, it does not provide significant insight as into why. One possibility is

that a single wild-type amino-terminal domain is required. Another possibility is that two amino-terminal domains (*i.e.* one wild-type and one chimeric) are required for nuclear translocation and subsequent deactivation. Yet another possibility is that Stat1 has a much more effective nuclear localization sequence under conditions of transient expression. Or, it is possible that the nuclear localization sequence resides between amino acids 125 and 135 and was disrupted during the generation of the chimeric STATs. Several of these possibilities are currently under evaluation and have yielded some preliminary results. Consistent with the transient assay studies, when CH2/1 is stably expressed in a Stat1-deficient cell line (46), it fails to translocate to the nucleus in response to an activating stimulus. Attempts to stably coexpress CH2/1 and Stat1 β have been unsuccessful. In other studies, a new Stat2/Stat1 chimera has been generated placing the site of fusion more distally (*i.e.* approximately amino acid 325 (47)). Consistent with CH2/1 (and CH5/1), when this new chimera is transiently expressed, it exhibits a prolonged pattern of activation. This mutant is, however, more difficult to activate. In yet another set of studies, the nuclear localization sequence of the SV40 large T antigen (48) was fused to either Stat1 or the chimeras. The nuclear localization sequence-wild-type protein continues to be deactivated normally, but the nuclear localization sequence-chimeric proteins (which now translocate to the nucleus) fail to be deactivated normally. This observation supports the possibility that a single wild-type amino-terminal domain is required for nuclear translocation.

In summary, these studies demonstrate that the generation of chimeric STATs leads to a relatively modest structural change, which blocks nuclear translocation and deactivation. The ability of wild-type Stat1 to rescue this defect suggests that at least one wild-type (*i.e.* "self") amino terminus is required for normal function. Further studies will be required to confirm this or other models and to determine whether a cell can distinguish wild-type from chimeric amino termini. These observations are, however, reminiscent of a regulatory "homotypic" (*i.e.* self) interaction mediated by the carboxyl- and amino-proximal termini of the Src family of tyrosine kinases (49).

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