Enhancement of Antiproliferative Activity of Gamma Interferon by the Specific Inhibition of Tyrosine Dephosphorylation of Stat1

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Gamma interferon (IFN- γ) signals to the nucleus through the activation, by tyrosine phosphorylation, of the latent cytoplasmic transcription factor Stat1 (signal transducer and activator of transcription). It has been demonstrated that the activity of Stat1 is dependent on tyrosine phosphorylation which is regulated by Jak tyrosine kinases as well as by the as-yet-unidentified protein tyrosine phosphatase. We report that the N-terminal domain of Stat1, which is highly conserved among all STAT family members, is required for its tyrosine dephosphorylation. A single amino acid substitution (Arg-31 to Ala) in the Stat1 N-terminal domain inhibited Stat1 tyrosine dephosphorylation. The deletion of the Stat1 N-terminal domain resulted in a mutant Stat1 protein which was constitutively phosphorylated on Tyr-701. Upon IFN- γ stimulation, the tyrosine phosphorylation of this mutant protein was further enhanced but was not down-regulated by protein tyrosine phosphatase *in vivo*. When expressed in NIH 3T3 cells, this mutant protein greatly enhanced the antiproliferative activity of IFN- γ . We suggest that the N-terminal domains of STATs are crucial for modulating STAT activities through regulating the tyrosine dephosphorylation of STATs.

Interferons (IFNs) have antiviral, immunomodulatory, and antiproliferative properties (8). A direct signal transduction pathway from the IFN receptor to the nucleus has been discovered (4, 22, 36, 45). The binding of IFN to its receptor leads to receptor dimerization and the activation of Janus kinase (Jak) tyrosine kinases (30, 41, 42, 46, 48). Specific tyrosine residues on the receptor are then phosphorylated by activated Jaks and serve as docking sites for recruiting a family of latent cytoplasmic transcription factors termed signal transducers and activators of transcription (STATs) (12, 13). The STATs are then phosphorylated on tyrosine, most likely by Jaks, and form homo- or heterodimers which subsequently translocate into the nucleus and direct immediate gene activation. It has been shown that gamma IFN (IFN- γ) activates the Stat1 homodimer, which binds to the IFN- γ response element and activates transcription (6, 38, 39).

STATs belong to a growing family of proteins involved in signal transduction by many cytokines and growth factors. Sequence analysis indicates that members of the STAT family of proteins are highly homologous in several regions. The SH2 domains of STATs have been shown to be crucial for both the activation and dimerization of STAT proteins (10, 38). It has been shown that the specificity of STAT activation is largely determined by the SH2 domains of STATs (16) as well as the specific STAT binding motifs present on ligand receptors (13, 43). A region of about 200 amino acid residues immediately before the SH3-like domain has been shown to be involved in mediating the DNA binding specificity of STATs (17). The transcription activation domain is located at the C terminus (31, 40, 49).

One distinct characteristic of STATs is that they are transcription factors whose activities are modified and regulated by tyrosine phosphorylation. Biochemical analysis has shown that tyrosine phosphorylation is crucial for the activity of STATs

(10, 14, 20, 35, 39). It has been found that Stat1 is phosphorylated on a single residue, Tyr-701, in response to stimulation by a number of ligands, including IFNs and epidermal growth factor (40, 41). The phosphorylation on the Tyr-701 residue of Stat1 has been shown to be required for its nuclear translocation, dimerization, DNA binding, and gene activation (38, 40). The tyrosine phosphorylation of STATs is regulated by Jak protein tyrosine kinases as well as the as-yet-unidentified protein tyrosine phosphatase (PTPase). It has been shown that a tyrosine phosphatase activity is involved in the down-regulation of IFN-induced gene expression (5, 15, 20, 21). Treatment with sodium vanadate, a PTPase inhibitor, can maintain the tyrosine phosphorylation of Stat1 and prevent the transcriptional down-regulation of IFN-induced genes (5, 21). Since the activities of STATs are absolutely dependent on tyrosine phosphorylation, the precise recognition and dephosphorylation of STATs by their PTPases are expected to be crucial for gene regulation. We report here that the Stat1 N-terminal domain, which is highly conserved among all STAT family members isolated so far, is required for the tyrosine dephosphorylation of Stat1. An N-terminal deletion mutant Stat1 protein was constitutively phosphorylated on tyrosine and greatly enhanced the antiproliferative activity of IFN- γ .

MATERIALS AND METHODS

Construction of plasmids and mutagenesis. pGST-Stat1, encoding GST-Stat1, was constructed by inserting Stat1 cDNA into the *Bam*HI site of the eukaryotic expression vector pEBG (2) (a gift from G. Cheng, Massachusetts Institute of Technology), which contains a glutathione *S*-transferase (GST) tag upstream of the cloning sites. pGST-mStat1, encoding GST-mStat1, was created by subcloning a PCR fragment encoding amino acids 62 to 750 of Stat1 into the *Bam*HI site of pEBG. pGST-myStat1 was derived from pGST-mStat1 in which the TAT codon for Tyr-701 was changed to TTT (for Phe). pGST-pStat1 was derived from pGST-Stat1 in which the AGA codon for Arg-31 was mutated to GCA (for Ala). For expressing the mutant Stat1 protein (Arg $\rightarrow$ Ala) without a GST tag, the corresponding cDNA was cloned into the *Bam*HI site of the pEBB vector. Mutagenesis was achieved by a standard PCR approach (25).

Transfection and establishment of cell lines. Transient transfection in 293 cells was performed as previously described (40). For stable transfection, expression constructs together with the vector pMLTR (a gift from G. Cheng) containing the *neo* resistance gene were introduced into NIH 3T3 cells. At 48 h after transfection, stable clones were selected in the presence of 0.5 mg of G418

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FIG. 1. Sequence alignment of the N-terminal regions of STATs. The sequences shown are from amino acid residues 1 to 82 of human Stat1 (34), 1 to 87 of human Stat2 (11), 1 to 82 of mouse Stat3 (53), 1 to 82 of mouse Stat4 (50, 53), 1 to 86 of sheep Stat5 (47), and 1 to 81 of human Stat6 (18, 33). Asterisks indicate invariant residues; dots indicate similar residues.

(Gibco BRL) per ml as previously described (40). Stable cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% donor bovine serum (Gibco BRL). Similar approaches were used for the transfection and selection of stable clones in U3A cells.

Gel mobility shift analysis. Gel mobility shift analysis was performed as previously described (24). The Stat1 binding site from the promoter of the human FcγRI receptor was used as the probe (32, 38).

Preparation of cell extracts, Western blotting (immunoblotting), and immunoprecipitation. Cells were washed with cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, and 3 μg of aprotinin per ml. Immunoprecipitation was performed as previously described (39). The immunoprecipitates were separated by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE) and transferred to a nitrocellulose filter. The blot was probed with a specific antiphosphotyrosine antibody (PY20; Transduction Laboratory). The same filter was stripped and reprobed with anti-Stat1. Murine IFN-γ (Gibco) and human IFN-γ (a gift from Amgen) were used at final concentrations of 500 U/ml and 5 ng/ml, respectively. Anti-GST was made against the whole GST protein. Anti-Stat1 and Anti-Stat1c were used as previously described (34, 35).

RESULTS

A Stat1 N-terminal deletion mutant protein is constitutively activated. Sequence comparison indicates that members of the STAT family of proteins are highly homologous in several regions. These include the previously defined SH2 and SH3-like domains (10). Another region of strong sequence homology among all known STAT proteins is at the N terminus (Fig. 1). In order to understand the function of STAT N-terminal domains, we constructed an expression vector encoding a mutant Stat1 protein lacking the N-terminal 61 amino acids. To distinguish the mutant protein from the endogenous Stat1β (the other form of Stat1, which lacks the C-terminal 38 amino acid residues present in Stat1α [34]), GST was fused to the N terminus of the mutant protein as a tag (GST-mStat1). As a control, an expression vector encoding the wild-type Stat1 with GST fused to the N terminus (GST-Stat1) was constructed (Fig. 2A). To determine if the N-terminal region of Stat1 is required for the activation of the Stat1 protein, GST-Stat1 and GST-mStat1 were transiently expressed in 293 cells (human embryonic kidney cells). Western blot analysis with anti-Stat1c antibody (an antibody against the C-terminal 38 amino acid residues of Stat1 [34]) indicated that both GST-Stat1 and GST-

mStat1 were overexpressed in 293 cells (Fig. 2B). The activities of the wild-type and mutant Stat1 proteins were then analyzed by gel mobility shift analysis with the Stat1 binding site from the promoter of the FcγRI gene as a probe (32, 38). As shown

FIG. 2. Analysis of the activity of the Stat1 N-terminal deletion mutant protein by transient transfection. (A) Diagram of expression constructs encoding GST-Stat1 and GST-mStat1. (B) Western blot analysis. Expression vectors encoding GST, GST-Stat1, and GST-mStat1 were transfected into 293 cells. Equal amounts of protein extracts were analyzed by SDS-7.5% PAGE and detected with anti-Stat1c. (C) Gel mobility shift analysis. Samples from panel B were analyzed by gel mobility shift analysis. -, no IFN treatment; +, IFN treatment.

FIG. 3. The Stat1 N-terminal deletion mutant is constitutively activated. (A) Western blot analysis of NIH 3T3-derived stable clones. Equal amounts of protein extracts from NIH 3T3 cells, 1K5 cells (NIH 3T3 cells expressing GST-Stat1), and 2K10 cells (NIH 3T3 cells expressing GST-mStat1) were analyzed by SDS-7.5% PAGE and transferred to nitrocellulose (13). The filter was probed with anti-Stat1. (B) Gel mobility shift analysis. Protein extracts from NIH 3T3 cells (lanes 1 and 2), 1K5 cells (lanes 3 to 6), and 2K10 cells (lanes 7 to 10) with or without IFN- γ treatment were analyzed by gel retardation analysis. For antibody inhibition analysis, anti-GST (lanes 5 and 9) and anti-Stat1 (lanes 6 and 10) were used at a final dilution of 1:30. The probe used was the Stat1 binding site from the promoter of the Fc γ RI receptor gene. (C) Phosphotyrosine blot analysis. GST-mStat1 (lanes 1 and 2) and GST-Stat1 (lanes 3 and 4) were immunoprecipitated from 2K10 cells (lanes 1 and 2) and 1K5 cells (lanes 3 and 4) untreated (lanes 1 and 3) or treated with IFN- γ for 15 min (lanes 2 and 4).

in Fig. 2C, both GST-Stat1 and GST-mStat1, as well as the endogenous Stat1, were activated and could bind to DNA in response to IFN- γ stimulation. These data indicate that the N-terminal region of Stat1 is not required for Stat1 activation. However, the N-terminal deletion mutant of Stat1 showed constitutive DNA binding activity (Fig. 2C, lane 2).

To further analyze the function of the Stat1 N-terminal domain, plasmids encoding GST-Stat1 and GST-mStat1 were transfected into NIH 3T3 fibroblasts, and stable clones expressing the corresponding proteins were selected. Western blot analysis with anti-Stat1 (an antiserum raised against amino acids 609 to 716 of Stat1 [34, 35]) showed that GST-Stat1 in

clone 1K5 and GST-mStat1 in clone 2K10 were expressed at levels similar to that of the endogenous Stat1 in NIH 3T3 cells (Fig. 3A).

We next tested the role of the Stat1 N-terminal region in the activation of Stat1. Protein extracts from 1K5, 2K10, and NIH 3T3 cells with or without IFN- γ treatment were prepared and analyzed by gel mobility shift analysis. In NIH 3T3 cells, Stat1 (both the α and β forms) was activated in response to IFN- γ treatment (Fig. 3B, lane 2). In 1K5 cells, GST-Stat1 was activated and migrated more slowly (because of the GST tag) than the endogenous Stat1 protein (Fig. 3B, lane 4). The identity of the GST-Stat1 shift complex was confirmed by using specific

antibodies. While a rabbit antiserum against GST (anti-GST) blocked the formation of the GST-Stat1 gel shift complex, the binding of the endogenous Stat1 was not affected by the same antiserum treatment (Fig. 3B, lane 5). The formation of both the GST-Stat1 and the endogenous Stat1 complexes was inhibited by anti-Stat1 antiserum (Fig. 3B, lane 6). A similar analysis was also carried out with GST-mStat1 in 2K10 cells. Consistent with the results from the transient-transfection analysis with 293 cells, GST-mStat1 appeared to bind to DNA constitutively and formed a distinct gel shift band in untreated 2K10 cells (Fig. 3B, lane 7). The DNA binding activity of GST-mStat1 could be further enhanced after IFN- γ stimulation (Fig. 3B, lane 8). To confirm that the constitutive gel shift complex in untreated 2K10 cells was due to the binding of GST-mStat1, antibody inhibition analysis was carried out. The gel shift complex in untreated 2K10 cells was inhibited by either anti-GST or anti-Stat1 antiserum (Fig. 3B, lanes 9 and 10).

Since the DNA binding activity of Stat1 is dependent on tyrosine phosphorylation (40), we reasoned that the deletion of the highly conserved N-terminal region of Stat1 might cause constitutive tyrosine phosphorylation of GST-mStat1. To test this hypothesis, phosphotyrosine blot analysis was performed. Protein extracts prepared from 2K10 and 1K5 cells with or without IFN- γ treatment were immunoprecipitated with anti-GST. The immunoprecipitated GST-mStat1 and GST-Stat1 were then analyzed by SDS-PAGE and probed with an antiphosphotyrosine antibody (PY20). A significant level of GST-mStat1 was found to be constitutively tyrosine phosphorylated (Fig. 3C, lane 1). IFN- γ treatment could further enhance the level of tyrosine phosphorylation of GST-mStat1 (Fig. 3C, lane 2). The wild-type GST-Stat1 was not tyrosine phosphorylated in untreated cells (Fig. 3C, lane 3), and the activation of GST-Stat1 by tyrosine phosphorylation was detected only after IFN- γ stimulation (lane 4). Similar amounts of proteins were present in all samples, as indicated by reprobing the blot with anti-Stat1 (Fig. 3C, lower panel). These results were consistent with our observation that GST-mStat1 but not GST-Stat1 was constitutively activated in 293 cells in transient-transfection analysis (Fig. 2). We conclude that the Stat1 N-terminal deletion mutant protein is constitutively activated.

The constitutive tyrosine phosphorylation of the Stat1 N-terminal deletion mutant protein is specific on Tyr-701. It has been shown previously that Stat1 is phosphorylated on a single tyrosine residue, Tyr-701, in response to IFN- γ stimulation (40). This ligand-dependent phosphorylation is required for Stat1 dimerization, nuclear translocation, DNA binding, and gene activation (38, 40). To demonstrate that the constitutive tyrosine phosphorylation of GST-mStat1 was a specific event and not due to nonspecific phosphorylation on other tyrosine residues, we mutated Tyr-701 in GST-mStat1 to Phe. This mutant protein (GST-myStat1) was stably expressed in NIH 3T3 cells. A representative clone, 28K10, overexpressing GST-myStat1 (Fig. 4A, lane 2) was used for further tests. Phosphotyrosine blot analysis indicated that GST-myStat1 was not constitutively tyrosine phosphorylated (Fig. 4B, lane 1). In addition, IFN- γ treatment did not induce tyrosine phosphorylation of GST-myStat1 (Fig. 4B, lanes 2 and 3). Furthermore, gel mobility shift analysis showed that this single amino acid mutation (Tyr-701 to Phe) in GST-mStat1 abolished the constitutive DNA binding activity of GST-mStat1 (Fig. 4C, lane 1). These results suggest that the constitutive and IFN- γ -inducible tyrosine phosphorylation of GST-mStat1 was specific on Tyr-701.

The N-terminal domain of Stat1 is required for tyrosine dephosphorylation. We have shown above that the deletion of

the N-terminal region of Stat1 resulted in the constitutive tyrosine phosphorylation of Stat1. We reasoned that in normal cells there exists a chemical equilibrium between tyrosine-phosphorylated and unphosphorylated Stat1, a process regulated by a low level of constitutively activated Jak tyrosine kinases and STAT PTPase(s). The equilibrium can be shifted toward the accumulation of tyrosine-phosphorylated Stat1 by either activating the Jak kinases (as in the case of ligand stimulation) or inhibiting the corresponding tyrosine phosphatases by use of inhibitors. If a mutant Stat1 protein is resistant to tyrosine dephosphorylation because of the lack of the recognition sequence for the corresponding tyrosine phosphatase, the basal level of tyrosine phosphorylation of this mutant protein may be elevated. To test whether the N-terminal region of Stat1 is required for its tyrosine dephosphorylation, the level of tyrosine-phosphorylated GST-mStat1 after IFN- γ stimulation for various time periods was analyzed. Protein extracts from NIH 3T3 cells, 1K5 cells, and 2K10 cells treated with IFN- γ for various time periods were prepared and analyzed by immunoprecipitation and phosphotyrosine blot analysis. The tyrosine phosphorylation of Stat1 from the parental NIH 3T3 cells reached a maximum after 15 min of IFN- γ treatment and then decreased thereafter (Fig. 5A, lanes 1 to 5). A similar kinetics of tyrosine phosphorylation of GST-Stat1 was also observed (Fig. 5A, lanes 6 to 10). In contrast, tyrosine-phosphorylated GST-mStat1 continued to accumulate over a period of 19 h after IFN- γ stimulation (Fig. 5A, lanes 11 to 15). These results indicate that the N-terminal region of Stat1 is required for its tyrosine dephosphorylation.

We next determined the DNA binding activity of GST-mStat1 after IFN- γ stimulation for various time periods. Protein extracts from 3T3, 1K5, and 2K10 cells were analyzed by gel mobility shift analysis. The DNA binding activity of the endogenous Stat1 in the parental 3T3 cells reached a maximum after 15 min of IFN- γ treatment and declined thereafter (Fig. 5B, lanes 1 to 5). GST-Stat1 as well as the endogenous Stat1 in 1K5 cells showed a similar kinetics of DNA binding activity (Fig. 5B, lanes 6 to 10). In contrast, the DNA binding activity of GST-mStat1 in 2K10 cells was found to be prolonged over the period of 19 h tested (Fig. 5B, lanes 11 to 15), correlating with the profile of tyrosine phosphorylation (Fig. 5A, lanes 11 to 15). We conclude that the N-terminal region of Stat1 is required for the down-regulation of Stat1 activity.

Effect of a PTPase inhibitor on the activity of the Stat1 N-terminal deletion mutant protein. It has been shown previously that pretreatment of cells with sodium vanadate (a PTPase inhibitor) followed by IFN- γ stimulation can enhance the tyrosine phosphorylation and the DNA binding activity of Stat1 (5, 15, 20, 21). This enhanced activity is believed to be due to the inhibition of STAT PTPase activity by vanadate, resulting in the accumulation of more tyrosine-phosphorylated Stat1. We reasoned that if mStat1 is a mutant protein which is resistant to STAT PTPase action, vanadate treatment should have no effects on the DNA binding activity of mStat1. To test this hypothesis, 2K10 cells as well as the parental NIH 3T3 cells were pretreated with sodium vanadate for 10 min and then treated with IFN- γ for 15 min. Consistent with the previously reported results, gel mobility shift analysis showed that the DNA binding activity of the endogenous Stat1 in both NIH 3T3 and 2K10 cells was induced after IFN- γ stimulation (Fig. 6, lanes 2 and 5) and was further enhanced when cells were pretreated with vanadate (lane 3 and 6). In contrast, although the DNA binding activity of GST-mStat1 was increased in response to IFN- γ , no further enhancement was observed with vanadate treatment (Fig. 6, lane 3). These results support the

FIG. 4. The constitutive tyrosine phosphorylation of the Stat1 N-terminal deletion mutant is specific on Tyr-701. (A) Western blot analysis. Protein extracts from NIH 3T3 cells (lane 1) and 28K10 cells (lane 2) expressing GST-mStat1 which contains a substitution of Tyr-701 with Phe in GST-mStat1 were analyzed by Western blotting. The antibody used was anti-Stat1. (B) Phosphotyrosine blot analysis. GST-mStat1 and GST-mStat1 were immunoprecipitated from 28K10 cells (lanes 1 to 3) and 2K10 cells (lanes 4 and 5) with or without IFN- γ treatment and were separated by SDS-7.5% PAGE. The proteins were then transferred onto a nitrocellulose filter and blotted with a specific antiphosphotyrosine antibody (PY20; Transduction Laboratory) (lanes 1 to 5). The antibody used for immunoprecipitation was anti-GST. The same blot was stripped and reprobed with anti-Stat1 (lanes 6 to 10). (C) Gel mobility shift analysis. Protein extracts from 28K10 cells (lanes 1 to 3) and 2K10 cells (lanes 4 and 5) were analyzed by gel mobility shift assays. The probe used was the Stat1 binding sequence from the promoter of the Fc γ RI receptor.

introduced into the Stat1 N-terminal region. This amino acid residue is conserved in all STAT proteins isolated so far (Fig. 1). The expression construct encoding GST-Stat1 with this point mutation (GST-pStat1) was transfected into NIH 3T3 cells. A representative clone, 31K2, expressing GST-pStat1 was used for further analysis. Protein extracts from 31K2 cells treated with IFN- γ for various period of time were prepared. These extracts were used for immunoprecipitation with anti-Stat1c antiserum. The immunoprecipitates were analyzed by SDS-PAGE and blotted with a specific antiphosphotyrosine antiserum (PY20; Transduction Laboratory). It was shown that the IFN-induced tyrosine phosphorylation of GST-pStat1 was prolonged (Fig. 7A). In contrast, the tyrosine phosphorylation of the endogenous Stat1 reached a maximum after 15 min of IFN- γ treatment and declined thereafter. Similar amounts of proteins were present in each lane, as shown by reprobing the blot with anti-Stat1 (Fig. 7B).

To further demonstrate the importance of the Stat1 N-terminal domain in modulating the tyrosine dephosphorylation of Stat1, the Arg-31 mutant Stat1 protein (pStat1) was introduced into U3A cells without a GST tag. U3A cells do not contain the endogenous Stat1 protein (31). A cell line (20K17) expressing pStat1 was selected and used for further analysis (Fig. 7C). (Two small truncated Stat1 proteins [with sizes in the range of 65 to 75 kDa] were also detected in this cell line. Since these truncated proteins could be detected by the antibody raised against the C-terminal 38 amino acid residues of Stat1, they seem to lack a large portion of the N-terminal region. It has been previously reported that a mutant Stat1 protein with the deletion of 141 amino acid residues at its N terminus could not be tyrosine phosphorylated in response to IFN [23]). A Stat1-

conclusion that the Stat1 N-terminal deletion mutant protein is insensitive to PTPase action.

A single amino acid mutation in the Stat1 N-terminal domain inhibits the tyrosine dephosphorylation of Stat1. Our data presented above demonstrated that the N-terminal region of Stat1 is required for the tyrosine dephosphorylation of Stat1. The N-terminal regions of all STAT family members isolated so far are highly conserved, and there are several invariant residues present in this region. We reasoned that these conserved amino acid residues may be directly involved in interacting with STAT PTPases. To examine the importance of these conserved residues in mediating Stat1 tyrosine dephosphorylation, a single point mutation (Arg-31 $\rightarrow$ Ala) was

FIG. 5. The Stat1 N-terminal deletion mutant protein is defective in tyrosine dephosphorylation. (A) Whole-cell extracts from 3T3 cells (lanes 1 to 5), 1K5 cells (lanes 6 to 10), and 2K10 cells (lanes 11 to 15) treated with IFN- γ for 0 min, 7 min, 15 min, 4 h, and 19 h were subjected to immunoprecipitation with anti-GST antibody. The immunoprecipitates were analyzed by phosphotyrosine blot analysis with PY20 (upper panels). The same blots were stripped and reprobed with anti-Stat1 (lower panels). (B) Gel mobility shift analysis. The protein extracts from panel A were analyzed by gel mobility shift assays. The probe used was the Stat1 binding sequence from the promoter of the Fc γ RI receptor.

complemented U3A cell line, C91, was used as the control (40). C91 and 20K17 cells were treated with IFN- γ for various periods of time, and protein extracts were prepared and analyzed by gel mobility shift analysis. The activity of the wild-type Stat1 reached a maximum after 15 min of IFN treatment and declined thereafter (Fig. 7D, lanes 1 to 4). However, the activity of the Arg-31 mutant Stat1 protein in 20K17 cells was prolonged and was not down-regulated (Fig. 7D, lanes 5 to 8). The weak DNA binding activity of pStat1 detected in 20K17 cells is due to the low expression level of this mutant protein (Fig. 7C). This result is consistent with our analysis with GST-tagged pStat1. The finding that the conserved residue Arg-31 is

crucial for the tyrosine dephosphorylation of Stat1 further supports our conclusion that the N-terminal domain of Stat1 is required for tyrosine dephosphorylation. A similar analysis was done by mutating another conserved amino acid, Glu-39, to Ala in the N terminus of Stat1. This mutant protein was also shown to be insensitive to tyrosine dephosphorylation (data not shown).

Constitutively activated Stat1 protein enhances the antiproliferative activity of IFN- γ . Stat1 is a key signal transducer and transcriptional activator in the IFN- γ signal transduction pathway. We have shown above that the N-terminal deletion mutant of Stat1 was constitutively activated and that the further-

FIG. 6. Effect of sodium vanadate on the activation of the Stat1 N-terminal deletion mutant protein. Gel mobility shift analysis was performed with extracts from cells mock treated (lanes 1 and 4), treated with IFN- γ for 15 min (lanes 2 and 5), or pretreated with sodium vanadate for 10 min and then treated with IFN- γ for 15 min (lanes 3 and 6). For vanadate treatment, the combination of 1 mM vanadate and 2 mM H_2O_2 was used.

enhanced activation of this mutant protein in response to IFN- γ was prolonged. We reasoned that such a mutant Stat1 protein may likely affect the biological activities of IFN- γ . It is known that IFN- γ has powerful antiproliferative activity on a number of target cells, including hematopoietic and epithelial cells (7, 26). However, many cell lines are also found to be insensitive to the antiproliferative activity of IFNs. This dramatic host difference in susceptibility to the antigrowth activity of IFNs is not understood. To study the potential role of Stat1 in the antiproliferative activity of IFN- γ , we analyzed the growth and morphological properties of NIH 3T3 cells, 1K5 cells (expressing GST-Stat1), and 2K10 cells (expressing GST-mStat1). These cells were grown in the presence or absence of IFN- γ for 4 days. The growth of NIH 3T3 cells was insensitive to IFN- γ treatment at 500 U/ml (Fig. 8A), and the IFN- γ treatment did not induce a morphological change of NIH 3T3 cells either (Fig. 8B, panels a and b). 1K5 cells expressing the wild-type GST-Stat1 behaved similarly to the parental NIH 3T3 cells with regard to growth (Fig. 8A) and morphological properties (Fig. 8B, panels c and d) regardless of the absence or presence of IFN- γ . In contrast, we found that IFN- γ could dramatically inhibit the proliferation of 2K10 cells (Fig. 8A). After exposure to IFN- γ for 4 days, a large number of 2K10 cells were found to be rounded up and detached from the plate (Fig. 8B, panel f). Even in the absence of IFN- γ stimulation, 2K10 cells expressing GST-mStat1 displayed altered morphology, being rounded up and refractile (Fig. 8B, panel e). These cells also grew relatively slowly compared with the parental

NIH 3T3 cells as well as 1K5 cells expressing the wild-type GST-Stat1 (Fig. 8A). Similar results were observed for other independent clones (data not shown). Our finding that a constitutively activated Stat1 protein can greatly enhance the antiproliferative activity of IFN- γ strongly suggests that Stat1 plays an important role in transducing the antiproliferative signal from the IFN receptor.

DISCUSSION

The precise recognition and dephosphorylation of tyrosine-phosphorylated substrates by their corresponding phosphatases are crucial for the biological activities of these proteins (19, 44). How PTPases can specifically recognize and dephosphorylate target proteins is poorly understood. A number of studies suggest that the substrate specificity of a PTPase can be regulated in several ways (51, 52). The amino acid sequence surrounding the site of phosphorylation and dephosphorylation of a target protein can affect the specific activity of the corresponding PTPase. The subcellular localization of a PTPase can also play an important role in controlling its substrate specificity. Our data presented here suggest that the N-terminal domain of Stat1 is required for the specific tyrosine dephosphorylation of Stat1. This conclusion is supported by several pieces of evidence. First, the tyrosine phosphorylation of the Stat1 N-terminal deletion mutant protein was prolonged over a period of 19 h after IFN stimulation and could not be down-regulated by PTPase. Second, the level of tyrosine-phosphorylated N-terminal deletion mutant Stat1 protein was not sensitive to treatment with vanadate, a PTPase inhibitor. Third, mutating invariant amino acid residues (Arg-31 to Ala or Glu-39 to Ala) in the N-terminal region inhibited the tyrosine dephosphorylation of Stat1. Since the N-terminal regions of all STAT family members are highly conserved, it is likely that they may function similarly in mediating the specific tyrosine dephosphorylation of STATs.

The Stat1 N-terminal domain involved in mediating the tyrosine dephosphorylation has not been precisely mapped. Interestingly, we did not detect a significant level of constitutive tyrosine phosphorylation of the mutant Stat1 protein with a single point mutation (Arg-31 to Ala). However, the deletion of the entire 61 amino acid residues showed a more dramatic effect, with the protein clearly being constitutively phosphorylated. It seems likely that a number of conserved amino acid residues at the N terminus are involved in mediating the interaction of Stat1 with its PTPase. It has been previously reported that a mutant Stat1 protein with a deletion of 141 amino acid residues at its N terminus was not tyrosine phosphorylated in response to IFN stimulation (23). It is likely that amino acid residues 60 to 141 may be involved in the activation of the Stat1 protein.

What is the role of the N-terminal domain in the specific tyrosine dephosphorylation of Stat1? One possible model for Stat1 tyrosine dephosphorylation is that the first step for the dephosphorylation of Stat1 is through the precise contact of a specific PTPase with Stat1 via specific protein-protein interactions. The N-terminal domain of Stat1 may serve directly as the recognition domain for the Stat1 PTPase (or for an adapter protein which recruits STAT PTPase). The specific dephosphorylation of Stat1 is achieved through the recruitment of its PTPase by the N-terminal domain. Alternatively, the N-terminal domain may work indirectly in mediating the tyrosine dephosphorylation of Stat1. The Stat1 N-terminal domain may not be the recognition domain for its PTPase. It is possible that the exposure of the as-yet-unidentified PTPase recognition region requires the presence of the N-terminal domain. The

FIG. 7. A single point mutation in the Stat1 N-terminal domain inhibits the tyrosine dephosphorylation of Stat1. (A) Phosphotyrosine blot analysis. 31K2 cells expressing GST-pStat1 (Arg-31→Ala) were treated with IFN- γ for various time periods as indicated. Equal amounts of protein extracts were used for immunoprecipitation (IP) with anti-Stat1c (an antiserum raised against the C-terminal 38 amino acids of Stat1). The immunoprecipitates were analyzed by SDS-7.5% PAGE and blotted with an antiphosphotyrosine antibody (PY20). (B) The same blot was stripped and reprobed with anti-Stat1 antiserum. (C) Western blot analysis. Protein extracts from a Stat1-complemented U3A cell line (C91) and a pStat1 (Arg-31→Ala)-complemented U3A cell line (20K17) were subjected to Western blot analysis. The blot was probed with anti-Stat1c. (D) Gel mobility shift analysis. Protein extracts prepared from C91 and 20K17 cells treated with IFN- γ for indicated time periods were analyzed by gel mobility shift analysis with the Stat1 binding sequence from the promoter of the Fc γ RI receptor as the probe.

deletion of or mutations in the Stat1 N-terminal domain may result in a change of protein conformation in which the actual Stat1 PTPase recognition region is buried and no longer accessible for the Stat1 PTPase. However, our data obtained so far argue for the first possibility. The fact that the Stat1 N-terminal deletion mutant protein can become tyrosine phosphorylated and bind to DNA suggests that a dramatic change of protein conformation is unlikely. Furthermore, single point mutations (Arg-31→Ala or Glu-39→Ala) in the N-terminal region also affect Stat1 tyrosine dephosphorylation. Finally, we have recently isolated a novel protein which interacts with the N-terminal region of Stat1. A segment of this protein shows strong homology with a highly conserved region found in many

PTPases (27). Although the exact role of the N-terminal domain in mediating the tyrosine dephosphorylation of Stat1 is not known at present, our data clearly show that the N-terminal domain is required for Stat1 tyrosine dephosphorylation. These findings point to a new direction for us to study the regulatory mechanism for STAT tyrosine dephosphorylation.

Recently, Stat1 knockout mice have been generated. These mice showed normal growth but were highly sensitive to infection by microbial pathogens and viruses (9, 28). These data demonstrated that the Jak-Stat1 pathway functions specifically in IFN action. Interestingly, studies have shown that the dysregulation of the Jak-STAT pathways is associated with a number of tumors. Constitutively activated Jak-STAT pathways

FIG. 8. Growth and morphological properties of NIH 3T3-derived cell lines in response to IFN- γ . (A) Analysis of the growth rates of NIH 3T3-derived cell lines. Parental NIH 3T3 cells, 1K5 cells (expressing GST-Stat1), and 2K10 cells (expressing GST-mStat1) were plated in 60-mm-diameter culture dishes at an initial cell density of 2×10^4 cells per ml. Murine IFN- γ (Gibco BRL) was added at a final concentration of 500 U/ml on day 0. Cell proliferation was determined over a period of 4 days by counting viable cells, as determined by trypan blue exclusion. The data are from one representative experiment. -IFN, no IFN- γ added; +IFN, IFN- γ added. (B) Morphological characteristics of NIH 3T3-derived cell lines. Parental NIH 3T3 cells (a and b), 1K5 cells (c and d), and 2K10 cells (e and f) were grown in the presence (b, d, and f) or absence (a, c, and e) of IFN- γ as described for panel A. The morphological characteristics of these cells at day 4 after the initial plating are shown.

have been observed in human T-cell leukemia virus type 1-mediated T-cell transformation (29) and in leukemia (1, 3, 37). The Stat1 N-terminal deletion mutant was found to be constitutively phosphorylated on Tyr-701. This mutant protein greatly enhanced the antiproliferative activity of IFN. The N-terminal regions of STAT proteins are highly homologous, and they may function similarly in mediating the tyrosine dephosphorylation of STATs. The ability to generate constitutively activated STAT proteins will allow us to dissect the biological functions of STATs.

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REFERENCES

- Carlesso, N., D. A. Frank, and J. D. Griffin. 1996. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J. Exp. Med.* **183**:811-820.
- Cheng, G., Z. S. Ye, and D. Baltimore. 1994. Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction. *Proc. Natl. Acad. Sci. USA* **91**:8152-8155.
- Danial, N. N., S. Pernis, and P. B. Rothman. 1995. Jak-STAT signaling induced by the v-abl oncogene. *Science* **269**:1875-1877.
- Darnell, J. E., Jr., I. M. Kerr, and G. M. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFN's and other extracellular signaling proteins. *Science* **264**:1415-1421.
- David, M., P. M. Grimley, D. S. Finbloom, and A. C. Lerner. 1993. A nuclear tyrosine phosphatase downregulates interferon-induced gene expression. *Mol. Cell. Biol.* **13**:7515-7521.
- Decker, T., D. J. Lew, J. Mirkovitch, and J. E. Darnell, Jr. 1991. Cytoplasmic activation of GAF, an IFN-gamma regulated DNA binding factor. *EMBO J.* **10**:927-932.
- Deiss, L. P., E. Feinstein, H. Berissi, O. Cohen, and A. Kimchi. 1995. Identification of a novel serine threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* **9**:15-30.
- De Maeyer, E., and J. De Maeyer-Guignard. 1988. Interferons and other regulatory cytokines. John Wiley and Sons, New York.
- Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84**:443-450.
- Fu, X.-Y. 1992. A transcriptional factor with SH2 and SH3 domains is directly activated by an interferon α -induced cytoplasmic protein tyrosine kinase(s). *Cell* **70**:323-335.
- Fu, X.-Y., C. Schindler, T. Improt, R. Aebersold, and J. E. Darnell, Jr. 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc. Natl. Acad. Sci. USA* **89**:7840-7843.
- Greenlund, A. C., M. A. Farrar, B. L. Viviano, and R. D. Schreiber. 1994. Ligand-induced IFN-gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system. *EMBO J.* **13**:1591-1600.
- Greenlund, A. C., M. O. Morales, B. L. Viviano, H. Yan, J. Krolewski, and R. D. Schreiber. 1995. Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. *Immunity* **2**:677-687.
- Gutch, M. J., C. Daly, and N. C. Reich. 1992. Tyrosine phosphorylation is required for activation of an alpha-interferon-stimulated transcription factor. *Proc. Natl. Acad. Sci. USA* **89**:11411-11415.
- Haque, S. J., V. Flati, A. Deb, and B. R. G. Williams. 1995. Roles of protein-tyrosine phosphatases in Stat1alpha-mediated cell signaling. *J. Biol. Chem.* **270**:25709-25714.

16. Heim, M. H., I. M. Kerr, G. R. Stark, and J. E. Darnell, Jr. 1995. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. *Science* **267**:1347–1349.
17. Horvath, C. M., Z. Wen, and J. E. Darnell, Jr. 1995. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev.* **9**:984–994.
18. Hou, J., U. Schindler, W. J. Henzel, S. C. Wong, and S. L. McKnight. 1995. Identification and purification of human Stat proteins activated in response to interleukin-2. *Immunity* **2**:321–329.
19. Hunter, T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**:225–236.
20. Igarashi, K., M. David, D. S. Finbloom, and A. C. Lerner. 1993. In vitro activation of the transcription factor gamma interferon-activating factor by gamma interferon: evidence for a tyrosine phosphatase/kinase signaling cascade. *Mol. Cell. Biol.* **13**:1634–1640.
21. Igarashi, K. I., M. David, A. C. Lerner, and D. S. Finbloom. 1993. In vitro activation of a transcription factor by gamma interferon requires a membrane-associated tyrosine kinase and is mimicked by vanadate. *Mol. Cell. Biol.* **13**:3984–3989.
22. Ihle, J. N. 1995. Cytokine receptor signaling. *Nature (London)* **377**:591–594.
23. Improt, T., C. Schindler, C. M. Horvath, I. M. Kerr, G. R. Stark, and J. E. Darnell, Jr. 1994. Transcription factor ISGF-3 formation requires phosphorylated Stat91 protein, but Stat113 protein is phosphorylated independently of Stat91 protein. *Proc. Natl. Acad. Sci. USA* **91**:4776–4780.
24. Kahn, K. D., K. Shuai, G. Lindwall, S. E. Maher, J. E. Darnell, Jr., and A. L. M. Bothwell. 1993. Induction of the Ly-6A/E gene by both interferon alpha/beta and gamma requires a DNA element to which a tyrosine phosphorylated 91 kDa protein binds. *Proc. Natl. Acad. Sci. USA* **90**:6806–6810.
25. Kawasaki, E. 1990. Recombinant PCR, p. 117–183. *In* M. Innis, D. Gelfand, J. Sninsky, and T. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
26. Kimchi, A. 1992. Cytokine triggered molecular pathways that control cell cycle arrest. *J. Cell. Biochem.* **50**:1–9.
27. Liao, J., and K. Shuai. Unpublished data.
28. Meraz, M. A., J. M. White, K. C. F. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, and J. K. Riley. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**:431–442.
29. Migone, T.-S., J.-X. Lin, A. Cereseto, J. C. Mulloy, J. J. O'Shea, G. Franchini, and W. J. Leonard. 1995. Constitutively activated Jak-STAT pathway in T-cells transformed with HTLV-1. *Science* **269**:79–81.
30. Muller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, S. Pellegrini, A. F. Wilks, J. N. Ihle, G. R. Stark, and I. M. Kerr. 1993. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature (London)* **366**:129–135.
31. Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improt, J. E. Darnell, Jr., G. R. Stark, and I. M. Kerr. 1993. Complementation of a mutant cell line: central role of the 91-kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathway. *EMBO J.* **12**:4221–4228.
32. Pearce, R. N., R. Feinman, K. Shuai, J. E. Darnell, Jr., and J. V. Ravetch. 1993. Interferon gamma-induced transcription of the high-affinity Fc receptor of IgF requires assembly of a complex that includes the 91-kDa subunit of transcription factor ISGF3. *Proc. Natl. Acad. Sci. USA* **90**:4314–4318.
33. Quelle, F. W., K. Shimoda, W. Thierfelder, C. Fishner, A. Kim, S. M. Ruben, J. L. Cleveland, J. H. Pierce, A. D. Keegan, K. Nelms, et al. 1995. Cloning of murine Stat6 and human Stat6, Stat proteins that are tyrosine phosphorylated in responses to interleukin-4 (IL-4) and IL-3 but are not required for mitogenesis. *Mol. Cell. Biol.* **15**:3336–3343.
34. Schindler, C., X.-Y. Fu, T. Improt, R. Aebersold, and J. E. Darnell, Jr. 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91 and 84 kDa ISGF-3 proteins that are activated by interferon-alpha. *Proc. Natl. Acad. Sci. USA* **89**:7836–7839.
35. Schindler, C., K. Shuai, V. R. Prezioso, and J. E. Darnell, Jr. 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* **257**:809–815.
36. Shuai, K. 1994. Interferon-activated signal transduction to the nucleus. *Curr. Opin. Cell Biol.* **6**:253–259.
37. Shuai, K., J. Halpern, J. ten Hoeve, X. Rao, and C. L. Sawyers. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. *Oncogene*, in press.
38. Shuai, K., C. M. Horvath, L. H. Tsai-Huang, S. Qureshi, D. Cowburn, and J. E. Darnell, Jr. 1994. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* **76**:821–828.
39. Shuai, K., C. Schindler, V. R. Prezioso, and J. E. Darnell, Jr. 1992. Activation of transcription by IFN- γ : tyrosine phosphorylation of a 91 kD DNA binding protein. *Science* **259**:1808–1812.
40. Shuai, K., G. R. Stark, I. M. Kerr, and J. E. Darnell, Jr. 1993. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* **261**:1744–1746.
41. Shuai, K., A. Ziemiecki, A. F. Wilks, A. G. Harpur, H. B. Sadowski, M. Z. Gilman, and J. E. Darnell, Jr. 1993. Polypeptide signaling to the nucleus through tyrosine phosphorylation of JAK and STAT proteins. *Nature (London)* **366**:580–583.
42. Silvennoinen, O., J. N. Ihle, J. Schlessinger, and D. E. Levy. 1993. Interferon-induced nuclear signaling by Jak protein tyrosine kinases. *Nature (London)* **366**:583–585.
43. Stahl, N., T. J. Farruggella, T. G. Boulton, Z. Zhong, J. E. Darnell, Jr., and G. D. Yancopoulos. 1995. Choice of Stats and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* **267**:1349–1353.
44. Sun, H., and N. K. Tonks. 1994. The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trends Biochem. Sci.* **19**:480–485.
45. Taniguchi, T. 1995. Cytokine signaling through nonreceptor protein tyrosine kinase. *Science* **268**:251–255.
46. Velazquez, L., M. Fellows, G. R. Stark, and S. Pellegrini. 1992. A protein tyrosine kinase in the interferon alpha/beta signalling pathway. *Cell* **70**:313–322.
47. Wakao, H., F. Gouilleux, and B. Groner. 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.* **13**:2182–2191.
48. Watling, D., D. Guschin, M. Muller, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, G. R. Stark, and I. M. Kerr. 1993. Complementation of a mutant cell line defective in the interferon-gamma signal transduction pathway by the protein tyrosine kinase Jak2. *Nature (London)* **366**:166–170.
49. Wen, Z., Z. Zhong, and J. E. Darnell, Jr. 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**:241–250.
50. Yamamoto, K., F. W. Quelle, W. E. Thierfelder, B. L. Kreider, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, O. Silvennoinen, and J. N. Ihle. 1994. Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. *Mol. Cell. Biol.* **14**:4342–4349.
51. Zhang, Z. Y., and J. E. Dixon. 1994. Protein tyrosine phosphatases: mechanism of catalysis and substrate specificity. *Adv. Enzymol. Relat. Areas Mol. Biol.* **68**:1–36.
52. Zhang, Z.-Y., A. M. Theime-Seffer, D. Maclean, D. J. McNamara, T. K. Sawyer, and J. E. Dixon. 1993. Substrate specificity of the protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA* **90**:4446–4450.
53. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1995. Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc. Natl. Acad. Sci. USA* **91**:4806–4810.