Interleukin 4 Regulates Phosphorylation of Serine 756 in the Transactivation Domain of Stat6

ROLES FOR MULTIPLE PHOSPHORYLATION SITES AND Stat6 FUNCTION*

Received for publication, December 15, 2003, and in revised form, March 16, 2004
Published, JBC Papers in Press, April 6, 2004, DOI 10.1074/jbc.M313668200

Yuling Wang‡, Maria Grazia Malabarba§, Zsuzsanna S. Nagy¶, and Robert A. Kirken‡

From the ‡Department of Integrative Biology and Pharmacology, The University of Texas Medical School, Houston, Texas 77030 and the §Department of Experimental Oncology, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy

Lymphokines interleukin-4 (IL4) and IL13 exert overlapping biological activities via the shared use of the IL4 receptor α-chain and signal transducer and activator of transcription 6 (Stat6). Stat6 is critical for T helper 2 cell differentiation, B-cell Ig class switch, and allergic diseases; thus, understanding its regulation is of central importance. Phosphorylation is crucial for Stat activity. Whereas Stat6 is phosphorylated on Tyr641, less is known about serine or threonine. We demonstrate in primary human T-cells (>95% CD3+) that IL4 and for the first time IL13 induce Stat6 serine but not threonine phosphorylation that closely paralleled early IL4 receptor α-chain activation (10 min). Stat6 uniquely fails to share a positionally conserved Stat serine phosphorylation sequence; however, known phosphoacceptor sites are proline-flanked. Alanine substitutions of these conserved residues revealed that the transactivation domain, which localized Ser756 but not Ser527 or Ser175, is the IL4-regulated site based on phosphoamino acid analysis. Tyr641 was dispensable for IL4-mediated serine phosphorylation, suggesting that dimerization is not preconditional. Only Stat6 Y641F variant showed a significant effect on IL4-inducible Ce DNA-binding and reporter gene expression. Lastly, recent work has shown that protein phosphatase 2A negatively regulates Stat6 (Woetmann, A., Brockdorff, J., Lovato, P., Nielsen, M., Leick, V., Rieneck, K., Svejgaard, A., Geisler, C., and Odum, N. (2003) J. Biol. Chem. 278, 2787-2791). We propose this target residue(s) is distinct from Ser756 and may be proximal to Tyr641 at Thr645, a residue conserved only among Stat6 members. The phosphomimic variants T645E or T645D ablated Stat6 activation, whereas polar uncharged substitutions (Gln or Asn) and additional mutants (Ala, Val, or Phe) showed no effect. These findings suggest that Stat6 has mechanisms of regulation distinct from other Stats.

1 This work was supported in part by Grants AI053566 and NIDDK 38016-12 from the American Lung Association, National Institutes of Health (to R. A. K.) and a grant from the American Heart Association, Texas Affiliate of the National Institutes of Health (to Y. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: The Fondazione Italiana per la Ricerca sul Cancro Institute for Molecular Oncology, via Adanello 16, 20139 Milan, Italy.

‡ To whom correspondence should be addressed: Dept. of Integrative Biology and Pharmacology, University of Texas-Houston, Medical Science Bldg., Rm. 4.218, Houston, TX 77030. Tel.: 713-500-7516, Fax: 713-500-7444; E-mail: robert.a.kirken@uth.tmc.edu.

IL4 and IL13 are homologous pleiotropic lymphokines secreted by antigen activated T-cells that act on cells of hematopoietic, endothelial, dendritic, osteoblastic, and fibroblastic origins and are critical for driving T-helper 2 cell differentiation (1, 2). T-helper 2 cell overproduction is commonplace during allergic maladies including asthma, allergic rhinitis, and atopic dermatitis via their ability to activate and recruit monocytes, basophils, mast cells, and eosinophils (1, 3). The overlapping biological effects of IL4 and IL13 are probably derived from their shared use of the IL4 receptor α (IL4Rα) signaling chain and their ability to activate one or more members of the Janus tyrosine kinases, which subsequently result in the activation of secondary effector molecules, including signal transducer and activator of transcription 6 (Stat) (2, 4, 5).

Stat6, originally cloned as IL-4-activated transcription factor (4), is responsible for immunoglobulin class switch and positive and negative gene expression in lymphocytes and for promoting the aforementioned pathologies based on studies from mice made Stat6-deficient through homologous recombination (6–9). Stat6 is one of seven Stat family members that is postulated to be recruited to newly phosphorylated tyrosine residues within activated receptors in the cytoplasm via their Src homology 2 domains, subsequently becoming tyrosine phosphorylated by Janus kinase or Src enzymes on a single and positionally conserved residue that is believed critical for Stat dimerization, nuclear localization, and gene transcription (10, 11). However, recent evidence suggests this model by probing that Stats exist as preformed dimers in the absence of tyrosine phosphorylation (12).

Several Stats also have been shown to be serine-phosphorylated in response to cytokine stimulation (13). Mapped Stat phosphoserine sites are localized within proline-rich motifs within the transactivation domain. For Stats 1, 3, 4, 5a, and 5b, this site is positionally conserved, whereas substitution/deletion of this residue/region can affect gene transcription (14–21). Stat6 does not share a positionally conserved phosphoserine acceptor site with other Stat family members; however, it has been shown to be serine-phosphorylated in Ramos Burkitt’s lymphoma B-cells (22) and murine splenic B-cells (23) in response to IL4, but the identity of this site(s) has remained elusive.

Various effector molecules also have been identified to neg-
Monoclonal anti-His6 antibody (catalog number MMs-156R, BaBCO).

Stat6 (catalog number AX56, Advantex Bioreagents, Conroe, TX) or against peptides derived from the extreme C termini of murine forms of Stat6 signals that may be mediated in part through tyrosine phosphorylation (31).

To identify these putative phosphoacceptor residues, Stat6 variants were generated against evolutionarily conserved serine residues flanked by prolines and then assessed by metabolic labeling and phosphoamino acid analysis. Herein, we identify the IL4-inducible phosphorylation site and characterize its ability to bind DNA and regulate gene transcription. The first evidence that IL13 modulates Stat6 serine phosphorylation in primary human T-cells also is shown. Lastly, we propose that an unknown phosphoserine or threonine residue can negatively regulate Stat6 activity because calyculin A inhibition of the serine-threonine protein phosphatase 2A (PP2A) disrupts Stat6 function. Corticosteroids also can negatively affect Stat6 signals that may be mediated through tyrosine phosphorylation (31).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**

Human T-lymphocytes obtained from normal donors were purified as described previously (32) and maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mEq/l-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml), respectively at 37 °C with 5% CO2. T-lymphocytes were activated for 2 h with phytohemagglutinin (PHA) (1 μg/ml) and were subsequently made quiescent by washing and incubating for 24 h in RPMI 1640 medium containing 1% fetal calf serum before exposure to cytokines. Cells were stimulated with recombinant human IL4 or IL13 (catalog number 200-13, Pepro Tech or catalog number 213-11, R&D system), at 37 °C as indicated below. HEK293 and COS-7 cells obtained from ATCC were grown in the same medium in the absence of PHA. Approximately 1 × 10^6 T-cells or 1 × 10^5 transfected cells were then incubated with medium or 100 nM IL-4 or IL-13 at 37 °C as indicated in the corresponding figure legends. Cell pellets were frozen at −70 °C until use.

**Flow Cytometry**

PHA-activated T-lymphocytes were stained with fluorescein isothiocyanate-labeled clone HIT3a (anti-CD3), anti-CD56 for Natural Killer cells, anti-CD14 for monocytes, and phosphatidylethanolamine-labeled anti-CD19 for B-lymphocytes. Antibodies were purchased from BD Biosciences as described previously (32). Cells were analyzed with a FACScan flow cytometer (BD Biosciences).

**Solubilization of Membrane Proteins and Immunoprecipitation**

Cells were solubilized in 1% Triton X-100 lysis buffer (106 cells/ml) and clarified by centrifugation as described previously (33). Supernatants were incubated with 5 μg/ml polyclonal rabbit antisera raised against peptides derived from the extreme C termini of murine forms of Stat6 (catalog number AX56, Advantex Bioreagents, Conroe, TX) or monoclonal anti-His6 antibody (catalog number MM-659, Sigma, St. Louis, MO). Blots were Western blotted with monoclonal mouse antiphosphotyrosine (catalog number 05-321, UBI, 4010), anti-Stat6, IL4R (R&D Systems) or anti-His6, antibody at 1:1000 as indicated previously (32). For serine-phosphatase inhibitor experiments, cells were preincubated with ethanol as mock control or varying concentrations of calyculin A (catalog number C-5552, Sigma) as described in the figure legends. For all of the samples, total protein was determined by BCA method (Pierce).

**Stat6 Variants and Transfections**

Stat6 human clone and empty vector pcDNA3.1/GS were purchased from Invitrogen. Mutants of Stat6 were prepared using the QuikChange site-directed mutagenesis kit (catalog number 200518, Stratagene, La Jolla, CA) with oligonucleotide primers designed to alter serine residues to alanines, tyrosine to phenylalanine, or threonine to one of several amino acids. The following mutants of human Stat6 were generated: S176A (AGT to GCC); S756A (AGC to GCC); S827A (TCC to GCC); Y641F (TAT to TTT); and Thr465 (ACC) to Glu (GAA), Ala (GCC), Gln (CAA), Val (GTA), Phe (TTC), Asp (GAC), or Asn (AAT). Before use, the DNA sequence of each mutant was verified. Transfections were performed in either HEK293 or COS-7 cells by FuGENE 6 transfection reagent (catalog number 1-814-443, Roche Applied Science) using 2 μg of the Stat6 plasmids/subconfluent COS-7 or HEK293 in 100-mm dishes, and after 48 h, cells were stimulated with 100 nM recombinant human IL4 for 20 min at 37 °C and then immunoprecipitated as described above or subjected to luciferase assay.

**Luciferase and β-Galactosidase Assays**—HEK293 cells were transfected with 0.5 μg of Stat6 wild type (WT) or variant plasmids, 1 μg of a triple repeat of the Cε gene promoter linked to the pGL3 luciferase reporter vector (Promega, Madison, WI), and 0.1 μg of the pCH110 plasmid containing the β-galactosidase gene as described previously (18). After 32 h, cells were stimulated with 1 nM recombinant human IL4 for 16 h at 37 °C. Luciferase and β-galactosidase activities were determined using the Dual-Light kit according to the manufacturer’s instruction (catalog number BD100LP, Tropix). To correct for differences in transfection efficiencies, luciferase activities were normalized to the β-galactosidase values in each individual sample. The results presented are representative data minimally from three independent experiments performed in triplicate.

**Electrophoretic Mobility Shift Assay**

Transfected cells expressing Stat6 WT or variants were subsequently treated without or with 100 nM human IL4 for 15 min and pelleted by centrifugation, and nuclear extracts were isolated and stored at −70 °C as described previously (34). Nuclear extracts (5 μg) were reacted with a Stat6 DNA binding element Cε that had been labeled with [γ-32P]ATP (4). For supershift assays, nuclear extracts were preincubated with 1 μg of mouse isotype control or His6 antibody for 4 °C for 1 h. Samples were then incubated with probe for an additional 15 min at room temperature. The DNA-protein complexes were resolved on a 5% polyacrylamide gel containing 0.25% Triton X-100, 0.5% borate EDTA buffer for 1 h at 100 V. After the loading of samples, gels were run at room temperature for 2 h at 150 V. Gels were then dried by heating under vacuum and exposed to film at −70 °C.
RESULTS

IL4 and IL13 Induce Tyrosine and Serine Phosphorylation of Stat6 in PHA-activated Human T-cells—To investigate the biological regulation of Stat6 driven by IL4 or IL13 in T-lymphocytes, primary human T-cells were isolated and activated for 72 h with PHA, stained, and subjected to fluorescence-activated cell sorter analysis for contaminating Natural Killer cells (CD56), B-cells (CD19), and monocytes (CD14). T-cells (CD3/H11001) represented 95% of this cell population (Fig. 1A).

Because Stat6 may be recruited to the IL4R via three phosphorylated tyrosine residues (37–39), we first examined the receptor tyrosine phosphorylation driven by IL4 and IL13 from 0 to 60 min to determine the time point for maximal phosphorylation. The immunoprecipitated receptor was subsequently immunoblotted with antiphosphotyrosine antibodies. The IL4Ra attained maximal tyrosine phosphorylation levels by either cytokine that peaked at 5–10 min (Fig. 1B, lanes b and c and lanes h and i). In comparison, Stat6 recruitment and tyrosine phosphorylation of Tyr641 showed a slightly protracted phosphorylation profile with detectable phosphorylation observed within 2.5 min and measured over the entire 40-min time course. (Fig. 1C) (27, 28). IL4 was more robust in its ability to induce tyrosine phosphorylation of both effector molecules against equally loaded samples (Fig. 1B and C, lower panels).

Previous studies have shown that IL4 induced Stat6 serine phosphorylation in Ramos Burkitt’s lymphoma B cells (22) and...
murine splenic B-cells (23). To investigate whether IL4 or IL13 is competent to induce a Stat6 serine phosphorylation in primary human T-cells, cells were radiolabeled with \([^{32}P]\)orthophosphate. Given the above activation kinetics of the IL4R and Stat6, cells were stimulated with either IL4 or IL13 for 20 min. Cell lysates were immunoprecipitated with Stat6 antibodies and subjected to protein separation. A single Coomassie Blue-stainable protein band was observed in each lane with an apparent molecular mass of 113 kDa, which corresponded with the autoradiograph (Fig. 1D, lower panel).

Each band was excised and subjected to phosphoamino acid analysis. Autoradiography revealed that the Stat6 protein showed radiolabeled phosphate incorporation into tyrosine and serine, but not threonine residues, in response to IL4 or IL13 stimulation of primary T-cells (Fig. 1D, lanes b and c).

Effect of Alanine Substitution of Serines 176, 756, and 827 or Tyrosine 641 of Stat6 as Judged by Phosphoamino Acid Analysis—All of the growth factor-mediated Stat serine phosphorylation sites mapped to date are flanked by one or more proline residues (11). To investigate the location of the Stat6 serine phosphorylation site(s), we identified only three evolutionarily conserved residues that met the criteria. This included Ser176 found in the helical coiled-coil domain, which promotes protein-protein interaction, and two that localized to the transactivation domain (Ser756 and Ser827) as depicted in Fig. 2A. To determine whether these sites were the putative IL4-IL13-inducible serine sites, HEK293 or COS-7 cells that express functional IL4 receptors but low to undetectable levels of Stat6 were transiently transfected with expression plasmids for WT or mutant forms of Stat6. Cells were metabolically labeled with \([^{32}P]\)orthophosphate and stimulated with or without 100 nM IL4 (100 nM) for 20 min at 37 °C are shown in lower panels of A and B. These bands were excised and subjected to acid hydrolysis and thin layer electrophoresis, and phosphate incorporated into amino acids was visualized by autoradiography as shown in the upper panels. Representative data from two independent experiments are shown. Migrational positions of phosphoserine (p-Ser), phosphothreonine (p-Thr), or phosphotyrosine (p-Tyr) are indicated on the right.

**Fig. 2.** Overview of mutation sites in Stat6 and phosphoamino acid analysis of WT and mutant proteins. Panel A, schematic of Stat6 and relative location of conserved proline-flanked serine residues compared with domains for DNA binding, coiled-coil (CCD), linker (LD), Src homology 2, and transactivation domains (TAD). Autoradiographs of immunoprecipitated WT and mutant forms of Stat6 from HEK293 (B) and COS-7 (C) cells that had been \([^{32}P]\)orthophosphate-labeled and incubated without (-) or with (+) IL4 (100 nM) for 20 min at 37 °C are shown in lower panels of A and B. These bands were excised and subjected to acid hydrolysis and thin layer electrophoresis, and phosphate incorporated into amino acids was visualized by autoradiography as shown in the upper panels. Representative data from two independent experiments are shown. Migrational positions of phosphoserine (p-Ser), phosphothreonine (p-Thr), or phosphotyrosine (p-Tyr) are indicated on the right.
remains to be determined. Serine phosphorylation may affect the oligomeric state of Stat6 insufficient to promote DNA binding (Fig. 3, lanes k). Phosphorylation of Ser756 alone is not sufficient to induce Stat6 serine phosphorylation in the absence of tyrosine phosphorylation, whereas IL4 can preformed dimers in the absence of tyrosine phosphorylation yet cannot bind DNA (12). Here we show that, although IL4 can promote the presence of Stat6 via an His6-supershifting antibodies compared with murine isotype control antibody (ctrl) (lane d). Arrow indicates migrational location of Stat6. Representative data from three separate experiments are shown.

inhibition of serine phosphorylation that was reduced to background levels in both cell types while tyrosine phosphorylation was not affected. It is concluded from these studies that Ser756 is the IL4-regulated Stat6 serine phosphorylation site. Stat6 did show some variability in COS-7 versus HEK293 cells since IL4-inducible threonine phosphorylation was occasionally but not reproducibly detected (panel C, lanes a and b). Lastly, the mutation of the conserved Y641F retained the ability to become serine-phosphorylated in both cell lines, suggesting that tyrosine and serine phosphorylation events can be mutually exclusive.

Analysis of DNA Binding Activities of Stat6 Mutants—The ability of wild type protein and Stat6 mutants to bind to an oligonucleotide probe was evaluated (Fig. 3). Protein nuclear extracts isolated from IL4-stimulated HEK293-Stat6-transfected plasmids were found to be fully capable of forming DNA complexes with a 32P-labeled probe with the exception of the Y641F variant (lane l). These results suggest that unlike Tyr641 (27, 28), Ser756 phosphorylation is not essential for nuclear localization or Stat6 DNA binding (lane h). Recent work by Braunstein et al. (12) has shown that Stat1 and Stat3 exist as preformed dimers in the absence of tyrosine phosphorylation yet cannot bind DNA (12). Here we show that, although IL4 can induce Stat6 serine phosphorylation in the absence of tyrosine phosphorylation, phosphorylation of Ser756 alone is not sufficient to promote DNA binding (Fig. 3, lanes k and l). Whether serine phosphorylation may affect the oligomeric state of Stat6 remains to be determined.

Effect of Mutating Ser756, Ser756, Ser827, and Tyr641 on IL4-induced Transcription—Earlier work (27) shows that the truncated Stat6Δ677 variant, void of two of three proline-flanked serines, is critical for gene expression. To analyze the role of all of the conserved proline-juxtaposed serines on Stat6 transactivation potential including Ser756, luciferase reporter assays were performed utilizing the Ce gene promoter. A triple repeat of this Stat6 binding element was transfected into HEK293 and COS-7 cells along with the expression plasmids encoding the Stat6 variants. A constitutively expressed β-galactosidase gene was also included to compensate for differences in transfection efficiencies. Luciferase activity was measured in extracts of cells that had been incubated in the absence or presence of IL4 for 16 h (Fig. 4, A and B). Wild-type Stat6 consistently mediated a 2–3-fold induction of reporter gene expression in either cell type in response to IL4 stimulation. Mutant Stat6 S756A mediated a response comparable to that of Stat6-WT, whereas Stat6 Y641F was reduced to background levels. Within these cell types, we conclude that serine phosphorylation of Stat6 is not a requirement for Ce gene transcription and that IL4-inducible Ser756 phosphorylation in the absence of Tyr641 phosphorylation is not sufficient to rescue its transcriptional activity. These results are in contrast to earlier studies with Stat5, its closest relative in which the deletion of Stat5a-regulated serine site elevated DNA binding activity, suggesting that this site might function in the capacity of a negative regulator (40, 41). These findings imply different roles for phosphoserine in gene transcription by Stat5 and Stat6.

Inhibition of Protein Phosphatase 2A Does Not Affect Gel Mobility of Stat5-depleted IL4-inducible Phosphoacceptor Sites—Previous work has shown that inhibition of PP2A by calyculin A blocks Stat6 DNA binding activity in various T-cell lines, suggesting that this phosphatase negatively regulates Stat6 activity (30) via a serine-threonine phosphorylation site(s). A biophysical consequence of this blockade is retarded gel mobility of Stat6 on SDS-PAGE, commonly observed for proteins in which phosphosites are flanked by prolines including Stats (18, 19, 35). To determine whether the calyculin A-induced phosphorylation was applicable to Stat6 in HEK293...
cells, a stably expressed WT Stat6 cell line was generated and intact cells were treated subsequently with calyculin A for 40 min at 37 °C (Fig. 5A). Calyculin A treatment resulted in altered Stat6 electrophoretic mobility as low as 50 nM (lane b) with a maximal change observed at 200 nM (lane d). To determine whether this putative phosphorylation site(s) could be localized to one of the three conserved proline-flanked serine residues, HEK293 cells expressing Stat6 variants were treated with 200 nM calyculin A for 40 min and assayed as in panel A. 

As shown in Fig. 5B, from one of four representative experiments, regardless of the Stat6 variant WT (lane b), S176A (lane d), S756A (lane f), and S827A (lane h), all displayed altered electrophoretic mobility including Y641F (lane j). These findings suggested that PP2A does not solely target the IL4-inducible phosphoacceptor sites Ser756, Tyr641, or the conserved proline-flanked serines.
To determine the identity of this regulated site, the stable WT Stat6-expressing HEK293 cell line was pretreated with 200 nM calyculin A without cytokine and Stat6 column affinity was purified, separated on SDS-PAGE, and stained with Coomassie Blue. Stat6 samples that showed retarded gel mobility compared with untreated control were excised and subjected to trypsin digestion, peptide isolation, and identification of charged species via mass spectrometry as described under "Experimental Procedures." A scan of phosphate precursor ions at m/z 79.3 yielded a peak at 926.3 was singly charged and localized to the tryptic fragment spanning Gly<sup>646</sup>-Lys<sup>647</sup>. Tandem mass spectrum of the positively charged m/z 928.4 was acquired, suggesting that the peaks were consistent with phosphorylation on a threonine residue that could reside at Thr<sup>645</sup> (data not shown).

To determine whether this residue may be a putative site for PP2A causing a loss of Stat6 function (30), we made a phosphomimic Stat6 variant of T645E. As shown in Fig. 5C, CA-treated WT cells showed reduced Stat6 tyrosine phosphorylation (lane c) as compared with untreated cells (lane b). However the single negatively charged Stat6 T645E variant showed a further reduction in tyrosine phosphorylation in the presence of IL4 (lane e). Interestingly, the T645E mutant migrated slightly slower on the gel; however, the CA treatment could suprashif this variant (lane f), suggesting that suboptimal or secondary phosphorylation sites may be present within this transcription factor. Electrophoretic mobility shift assay analysis of Stat6 (panel D) showed that unlike untreated WT (lane b), neither untreated T645E cells (lane d) nor HEK293 cells expressing WT (lane e) or T645E (lane f) treated in the presence CA and IL4 were competent to bind the radiolabeled C<sub>e</sub> probe. This condition was also reflected in the loss of transcriptional activity for T645E variant compared with WT Stat6 (panel E) in which reporter activity of the C<sub>e</sub>-luciferase was reduced to unstimulated background levels. Taken together, these findings suggest that Thr<sup>645</sup> may act as a site of negative regulation for Stat6 by inhibiting tyrosine phosphorylation and DNA binding.

To determine whether the loss of IL4-mediated tyrosine phosphorylation of T645E was attributed to polar or steric considerations, several additional mutations were generated. As shown in Fig. 5F, when compared with IL4-stimulated HEK293 cells transiently expressing WT Stat6 (lane b), only phosphomimics T645E (lane d) and T654D (lane h) showed a loss of tyrosine phosphorylation, whereas polar uncharged corresponding amino acids species Gln (lane g) and Asn (lane f), respectively, had no effect. Additionally, the mutation of Thr<sup>645</sup> to Ala (lane l) or spatially similar Val (lane o) or the hydrophobic bulky Phe residue (lane q) failed to affect IL4-mediated Stat6 tyrosine phosphorylation.

**DISCUSSION**

Stat6 plays a key role in a variety immune-based diseases such as allergy but is also frequently found constitutively activated in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma (42). IL4 and IL13 exert their biological effects through Stat6 to drive cell growth, survival, or differentiation in a cytokine-inducible manner. However, we found that the mutation of S756A failed to show a significant and reproducible change in DNA binding, although a minor increase in DNA binding was occasionally observed for S756A (Fig. 3, lane h). To further clarify this issue, the Stat6 S756E variant to mimic phosphorylated Stat6 was generated; however, DNA binding and reporter activity were equivalent to wild type Stat6 (data not shown).

Stat6 contains a modular proline-rich transactivation domain, which exists in some transcription factors including BSAP (45) and EKLF (28, 46) that may act to recruit proteins involved in chromatin reorganization. Additionally, Stat6 has been shown to interact with other regulators including NF-κB (47, 48), CREB-binding protein, NcoA-1, a CREB-binding protein-associated member of the p160/steroid receptor coactivator family (49) and the glucocorticoid receptor. Whether these proteins may interact with phosphorylated Ser<sup>756</sup> remains to be determined. Additionally, the identity of the Stat6 serine kinase is not immediately known. Several Stat serine kinases have been proposed to phosphorylate the PMSP motif, a consensus mitogen-activated protein kinase phosphorylation sequence (50). Supportive evidence have shown that Stats coprecipitate with ERK1/2 (16, 52) and can be inhibited by MEK1/2 poisons (14, 51, 53) as well as inhibitors to phosphatidylinositol 3-kinase (55) and mammalian target of rapamycin (56) but not p38 and JNK (57–59). We performed similar studies using WT Stat6-transfected HEK293 cells and failed to see any changes in Stat6 serine phosphorylation in response to IL4 stimulation, results similar to two earlier studies (22, 23). A recent study (54) finds that the Stat5a Ser<sup>779</sup> residue found within a SP site can directly interact and be phosphorylated by Pak1 and stimulate β-casein promoter activity. Pak1 is not a proline-directed serine-threonine kinase, and any role in phosphorylating Stat6 in T-cells is not presently clear. However, IL4 stimulation of
HEK293 or COS-7 cells failed to show activation of Pak1 as measured by phosphoantibodies directed to the catalytic auto-phosphorylation site (Thr645, data not shown), whereas other family members were not tested.

Recent work has demonstrated that Stat6 cycles between active and inactive forms of the protein that is independent of new protein synthesis (51). One enzyme that can inactivate both active and inactive forms of the protein that is independent of new protein synthesis (51). One enzyme that can inactivate Stat6 is the serine-threonine phosphatase PP2A, which is inhibited by CA (30). While Wostmann et al. (30) reported that phosphoamino acid analysis of CA primarily protected Stat6 serine phosphorylation and to a lesser extent threonine, we found predominantly the phosphorylation of Thr645 in our cell line, HEK293. Interestingly, Thr645 is conserved in mouse, rat, and human Stat6 but not other Stat family members.

Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members.

Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members.

Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members.

Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members.

Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members. Ser756 probably is not a target of the phosphorylation in response to IL-4. It is tempting to speculate that phosphorylation of Ser756 in Stat6 is the serine-threonine phosphatase PP2A, which is conserved in mouse, rat, and human Stat6 but is not present in other Stat family members.
Interleukin 4 Regulates Phosphorylation of Serine 756 in the Transactivation Domain of Stat6: ROLES FOR MULTIPLE PHOSPHORYLATION SITES AND Stat6 FUNCTION

Yuling Wang, Maria Grazia Malabarba, Zsuzsanna S. Nagy and Robert A. Kirken

J. Biol. Chem. 2004, 279:25196-25203.
doi: 10.1074/jbc.M313668200 originally published online April 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313668200

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 59 references, 38 of which can be accessed free at http://www.jbc.org/content/279/24/25196.full.html#ref-list-1