The Conserved Leu-724 Residue Is Required for Both Serine Phosphorylation and Co-activator Recruitment for Stat1-mediated Transcription Activation in Response to Interferon-γ*

Received for publication, May 27, 2005, and in revised form, September 12, 2005 Published, JBC Papers in Press, October 27, 2005, DOI 10.1074/jbc.M505797200

Wei Sun†1, Wei Feng Xu1, Marylynn Snyder1, Wei He1, Hao Ho2, Lionel B. Ivashkiv3*, and J. Jillian Zhang1,2

From the 1Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, New York 10021, 2Arthritis and Tissue Degeneration Program, and 3Immunology Program, Hospital for Special Surgery, New York, New York 10021

The signal transducer and activator of transcription (STAT) proteins, a family of latent cytoplasmic transcription factors, become activated in response to extracellular ligand binding to cell surface receptors through tyrosine phosphorylation. Concurrently, a serine phosphorylation event in the transcription activation domain (serine 727 for Stat1) occurs. This serine phosphorylation is essential for the maximal transcription activity of Stat1. Here we show that, in addition to the Ser-727 residue and its phosphorylation, the conserved Leu-724 residue is also essential for gene activation mediated by Stat1. When Leu-724 is mutated to Ala, phosphorylation of Stat1 Ser-727 is defective both in vivo and in vitro. Surprisingly, we found a Stat1L724I mutant that lacks transcription activity despite normal Ser-727 phosphorylation. Further analyses show that Leu-724, as well as the phospho-Ser-727, are essential for the recruitment of the transcription co-activator CBP/p300 to the promoters of Stat1 target genes. Our results demonstrate that the conserved Leu-724 residue is a key residue that controls the maximal transcription activities of Stat1 in IFN-γ signaling.

The signal transducer and activator of transcription (STAT) family of transcription factors mediates a multitude of cytokine-regulated gene transcription (1–3). In response to ligand binding to cell surface receptors, the STATs are activated through phosphorylation, form dimers, enter the nucleus, and bind to specific DNA sequences for transcription activation. For IFN-γ signaling, Stat1 is predominantly activated (4). The essential physiological function of Stat1 is demonstrated by the Stat1 knock-out mice, which have impaired IFN-γ signaling resulting in a high susceptibility to pathogens, high frequency of tumor occurrence, and lack of expression of many IFN-induced proteins (5–8).

The main biological functions of the STATs are carried out at the level of transcription activation of thousands of different genes in response to stimulation by various ligands. The transcription activation domain (TAD) of STATs is located in the carboxyl-terminal end of the molecule (9). The STAT TADs can function as independent domains for gene activation by recruiting other proteins, such as the transcription co-activator CREB-response element-binding protein (CBP)/p300 (10–16). Unlike the rest of the STAT molecule, which shares extensive sequence and structural homologies (17–19), there is only one conserved sequence motif (LPMSP) present in the TADs of some of the STATs, such as Stat1, Stat3, and Stat4 (20). In addition to the well-studied tyrosine phosphorylation in STATs, the serine residue (Ser-727) in the LPMSP motif has also been shown to be required for the maximal transcription activities of Stat1, Stat3, and Stat4 (21–23). In vivo analyses of Stat1/S727 using knock-in mouse models showed that Stat1S727 is required for IFN-γ-dependent innate immunity in vivo (24), and Stat3S727 plays an essential role in postnatal survival and growth (25).

Several serine kinases, including CaMKII, p38 mitogen-activated protein kinase, extracellular signal-regulated kinase mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT pathways have been shown to be involved in the phosphorylation of Stat1 and Stat3 (26–30). However, it is not clear what structural elements determine the specificity between the different STATs and the serine kinases activated by the various cytokines. The biochemical function underlying the physiological importance of the phosphoserine and conserved LPMSP motif is not completely understood either.

In this report, we show that, in addition to Ser-727 and its phosphorylation, the conserved Leu-724 residue is also essential for gene activation mediated by Stat1. Leu-724 is required for the phosphorylation of Stat1S727 both in vivo and in vitro. In addition, we present a Stat1 mutant that lacks transcription activity despite normal Ser-727 phosphorylation. Further analyses show that Leu-724, as well as the phospho-Ser-727, are essential for the recruitment of the transcription co-activator CBP/p300 to the promoters of Stat1 target genes. Our results demonstrate that Leu-724 is the key regulatory element that controls the maximal transcriptional activity of Stat1.

MATERIALS AND METHODS

Cell Culture and Antibodies—U3A cells (provided by G. Stark, Cleveland Clinic Foundation Research Institute, Cleveland, OH, and I. Kerr, Imperial Cancer Research Foundation, London, UK) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% cosmic calf serum. 293T and HT1080 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (HyClone). Isolation of peripheral blood mononuclear cells from whole blood obtained from healthy volunteers was done as described previously (31) with a protocol approved by the institutional review board of the Hospital for Special Surgery (New...
Human monocytes were purified from peripheral blood mononuclear cells immediately after isolation by positive selection with anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotec). The purity of the monocytes was >97%, as verified by flow cytometry. Antibody against phosphoserine-Stat1 and acetyl-histone H4 were from Upstate Biotechnology. Antibodies against phosphotyrosine-Stat1 and -Stat3 were from Cell Signaling Technology. Antibody against the Stat1 amino terminus was from Transduction Laboratories. Antibodies against the p300, CaMKII, and Stat1 carboxy termini were from Santa Cruz Biotechnology. Recombinant human IFN-γ (hIFN-γ) was from Roche Applied Science and used at 5 ng/ml hIFN-γ for the lengths of time indicated in each experiment. CaMKIIα/β purified from rat brain was from Calbiochem.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitation experiments were performed as previously described (32). Specifically, 10⁷ cells were used for each precipitation reaction with the following antibodies: 2 μg of anti-Stat1C, 2 μg of anti-p300, or 2 μg of anti-acetyl-histone H4. ChIP PCR primer pair sequences for the human IRF-1 promoter are: 5'-CTTCGCCGCTAGCTACACAAAGG-3' and 5'-GCTCCTGGGTGGCCTGGTCCG-3'. Primers for GBP-1 promoter are: 5'-TGAGAACACTTAAAAACCTCCTCC-3' and 5'-TGCTTACACTTCTGTGC-3'.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides representing a STAT-1 binding sequence M67 (33) were labeled with 3²P by Klenow filling. A total of 1 ng of labeled probe was incubated with 5 μg of nuclear extracts at room temperature for 10 min. The samples were loaded on a 5% polyacrylamide gel and run at constant voltage (200 V) for 3 h in a cold room.

Lentiviral Transduction in Human Monocytes—The generation of viral stocks and transduction into human primary monocytes were done essentially as previously described (31, 34). Briefly, lentiviral plasmids containing Stat1 and packaging vectors were transfected into 293T cells in 10-cm plates. Viral supernatants were harvested 48 and 72 h after transfection and titered on HT1080 cells. Viral particles were spun down at 25,000 revolutions/min for 90 min and resuspended in RPMI 1640 medium and then added to the human primary monocytes at a ratio of 1:50 in the presence of 4 μg/ml polybrene. 48 h after transduction, the efficiency of transduction was monitored with fluorescence microscopy to measure green fluorescent protein expression and was typically >80%. The cells were then either untreated or treated with IFN-γ for 30 min followed by Western blotting analyses.

RNA Analyses—Total RNAs were prepared using TRIzol (Invitrogen). Real-time RT-PCR analyses were done on the ABI PRISM® 7900HT sequence detection system (Applied Biosystems) with the SYBR Green PCR kit from Applied Biosystems as instructed by the manufacturer. The relative level of expression (RE) for a specific gene was calculated according to the equation \( \text{RE}_{\text{n}} = \frac{2^{- \Delta \Delta Ct}}{2^{- \Delta \Delta Ct}} \)

\( \Delta Ct = \) Ct test gene – CtGAPDH, \( C(t) \), cycle threshold; \( n \), specific sample; 0, untreated wild type; test gene, IFN-1, GBP-1, etc. Primer pair sequences for the real-time RT-PCR are: hSOCS-1, 5'-TTGTTTAGCACCTTTAATCTA-3' and 5'-AGAGTGGAGTGGCTGGATGTTG-3'; hGBP-1, 5'-ATAAGTTGAGCTGGAAGGG-3' and 5'-ATGGCTGCTGTAGAACGGA-3'; hIRF-1, 5'-AAATCCCCGGGCTCATTG-3' and 5'-CTGCTCCTTCTCTYGTTTGTG-3'; and hGBP-1, 5'-AGAGGAGAGTGGAAGAAA-3' and 5'-AATGGCTCTTAAAGTGGC-3'.

**FIGURE 1. The conserved Leu-24 is required for maximal transcription activity of Stat1.** A, a schematic diagram of Stat1 and the sequence alignment of the transcription activation domains of Stat1, Stat3, and Stat4. Domain structures are according to Chen et al. (18). Stat1α, full-length form of Stat1 containing the transcription domain (TAD); Y, tyrosine 701; S, serine 727. B, real-time RT-PCR analyses of Stat1 target gene expression in response to IFN-γ. Stable cell lines containing wild type and the various indicated mutant Stat1 proteins were treated with IFN-γ for 3 h. Total RNA samples were isolated and analyzed by real-time RT-PCR with primers specific for IFN-1, SOCS-1, GBP-1, and GAPDH. 3–5 independent stable cell lines were analyzed 2–3 times for each mutant, and one representative result is shown. Data presented are normalized against the internal control of GAPDH and shown as the fold of increase over the value from untreated cells expressing Stat1 wild type protein. U3, Stat1-null U3A cells; WT, wild type; L, leucine 724 alanine; SA, serine 727 alanine; LI, leucine 724 isoleucine; SD, serine 727 aspartic acid. C, whole cell lysates from the individual cell lines were analyzed by Western blotting with anti-Stat1 or -Stat3 antibodies. The amount of endogenous Stat3 was used as a control.

were constructed as previously reported (20). Specific point mutations in Stat1 were generated with the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. To generate lentiviral plasmids containing Stat1, DNA fragments containing the RcCMV promoter and Stat1 cDNA were PCR-amplified from the RcCMV plasmids containing wild type or mutant Stat1 and cloned into the lentiviral vector FUGW (kindly provided by David Baltimore, California Technology Institute, Pasadena, CA).

Transfection Experiments—Transient transfections of U3A cells and 293T cells were done with the Lipofectamine 2000 method (Invitrogen) according to manufacturer’s instructions. Luciferase assays were per-
formed using the Dual-Luciferase reporter system according to the manufacturer’s instructions (Promega). All luciferase results were luciferase activities normalized against an internal control luciferase reporter of Renilla luciferase (Promega). For generation of stable clones, U3A cells were transfected with the calcium-phosphate transfection kit (Invitrogen) as instructed by the manufacturer. 24 h after transfection, the cells were plated into medium containing 1 mg/ml G418 for 6 days, and G418-resistant clones were screened by Western blotting.

RESULTS

The Conserved Leu-724 Residue Is Required for Stat1-mediated Transcription Activation in Response to IFN-γ—We have previously reported that the conserved Leu-724 residue is required for Stat1 activity in transient transfection assays (23). To understand the biochemical basis for the physiological importance of this conserved residue for Stat1-mediated transcription activation in IFN-γ signaling, we generated a panel of Stat1 mutants in the conserved Leu-724 or Ser-727 (Fig. 1A). The Stat1-null U3A cells (35) were reconstituted with these mutants to generate stable cell lines containing these various forms of Stat1 proteins (Fig. 1C). Multiple cell lines containing each mutant were analyzed for the expression levels of three IFN-γ-inducible Stat1 target genes, IRF-1, SOCS-1, and GBP-1, as well as an internal control gene GAPDH by real-time RT-PCR. Results of one representative experiment are shown in Fig. 1B. Very little expression of the three Stat1 target genes was detected in the U3A cells and Stat1-containing cells without IFN-γ treatment (Fig. 1B). After stimulation with IFN-γ for 3 h, the expression level of IRF-1 increased 15-fold, and the levels of SOCS-1 and GBP-1 increased by 3–4-fold in cells containing wild type or mutant STAT1, cultured for 2 days, stimulated with IFN-γ for 30 min, and analyzed by Western blotting. F and G, the levels of PS-Stat1, Stat1, and Stat3 in Fig. 2E were further quantitated by Quantity One software using the Gel Doc 2000 gel documentation system (Bio-Rad) and shown as bar graphs. The signals from the endogenous Stat1 in the untransfected primary cells (lanes 1 and 2) were subtracted to show the signals from the overexpressed Stat1. F, relative level of phosphoserine Stat1 normalized against the level of Stat1. G, relative level of Stat1 expression normalized against the endogenous Stat3.

FIGURE 2. Requirement of Leu-724 for phosphorylation of Stat1 Ser-727. A, stable cell lines containing the various indicated forms of Stat1 were treated with IFN-γ for 30 min. Whole cell lysates were analyzed by Western blotting with the indicated antibodies. PS, phospho-Ser-727 of Stat1; PY, phospho-Tyr-701 of Stat1. Multiple stable cell lines for each Stat1 mutant were analyzed, and one representative experiment is shown. The amount of endogenous Stat3 was used as a control. B and C, the levels of PS-Stat1, Stat1, and Stat3 in Fig. 2A were further quantitated by Quantity One software using the Gel Doc 2000 gel documentation system (Bio-Rad) and shown as bar graphs. B, relative level of phosphoserine Stat1 normalized against the level of Stat1. C, relative level of Stat1 expression normalized against the endogenous Stat3. D, electrophoretic mobility shift assays were performed with nuclear extracts from the various indicated cells untreated or treated with IFN-γ for 30 min and a 32P-labeled M67 Stat1 binding site. n.s., nonspecific signal from the extracts. E, primary human monocytes were transduced with the FUGW lentiviral vector or FUGW vectors containing wild type or mutant STAT1 cultured for 2 days, stimulated with IFN-γ for 30 min, and analyzed by Western blotting.

The results from the overexpressed Stat1 in the untransfected primary cells (lanes 1 and 2) were subtracted to show the signals from the overexpressed Stat1. F, relative level of phosphoserine Stat1 normalized against the level of Stat1. G, relative level of Stat1 expression normalized against the endogenous Stat3.
we mutated Leu-724 to isoleucine (Ile), which should have caused a smaller structural change at this position than the L724A mutation. However, Stat1L724I failed to activate any of the Stat1 target genes (Fig. 1B), suggesting a stringent structural requirement for Leu-724 at this position. To dissect the biochemical function of the phospho-Ser-727, we mutated Ser-727 to aspartic acid to mimic the negative charge brought by serine phosphorylation. However, S727D could not induce significant levels of expression of the Stat1 target genes (Fig. 1B), suggesting that, in the context of a chromatin promoter, the single negative charge brought by Asp-727 to Stat1 is not sufficient to activate transcription. Altogether, these results indicate that the Leu-724 residue, in addition to Ser-727, is also essential for Stat1-mediated gene activation in response to cytokine stimulation.

Requirement of Leu-724 for Stat1 Ser-727 Phosphorylation—To understand the molecular basis of the defects in the above mutants for Stat1-mediated gene activation, we first investigated the status of tyrosine and serine phosphorylation in these mutants in response to cytokine stimulation. Cells containing the various forms of Stat1 were first treated with IFN-γ to see the effect of these mutations on IFN-γ-induced phosphorylation of Stat1. Western blotting experiments were done using antibodies specific to phospho-Ser-727 and phospho-Tyr-701 of Stat1. IFN-γ treatment induced strong Tyr-701 phosphorylation in wild type Stat1 as well as in all the mutant Stat1 proteins, indicating that mutations of Leu-724 or Ser-727 do not affect IFN-γ-induced phosphorylation of Tyr-701 (Fig. 2A, second panel, lanes 4, 6, 8, 10, and 12). For the status of Ser-727 phosphorylation, wild type Stat1 had a significant level of Ser-727 phosphorylation in untreated cells, and the level of Ser-727 phosphorylation increased moderately after IFN-γ treatment (Fig. 2A, top panel, lanes 3 and 4; and B). However, the Stat1L724A mutant could not be phosphorylated on Ser-727 either in untreated condition or after treatment with IFN-γ (Fig. 2A, top panel, lanes 5 and 6; and B). This result indicates that this L724A mutation causes a specific defect in Ser-727 phosphorylation in Stat1. Interestingly, the Stat1L724I mutant, which contains minimal structural change at position 724 and is defective for gene activation (Fig. 1B), could still be phosphorylated on Ser-727 (Fig. 2A, top panel, lanes 9 and 10; and B), suggesting that Ser-727 phosphorylation alone is not sufficient for Stat1 transcription activity. None of the Ser-727 mutants had detectable levels of Ser-727 phosphorylation above the weak nonspecific signal (Fig. 2A, top panel, lanes 7, 8, 11, and 12; and B). Similar levels of Stat1 were expressed in these stable clones compared with the endogenous level of Stat3 (Fig. 2C). These mutations do not affect the nuclear translocation of Stat1 (data not shown). The ability of these mutants to bind to DNA was further tested by electrophoretic mobility shift assay. IFN-γ-induced binding to the Stat1 site M67 was detected for all mutants, and quantitation of the Stat1 band normalized to the nonspecific band showed a similar level of binding (Fig. 2D; quantitation data not shown). These results indicate that Leu-724 is important for IFN-γ-induced Ser-727 phosphorylation of Stat1. Furthermore, the L724A mutation also blocked UV irradiation-induced Ser-727 phosphorylation (28, 36) in Stat1 (data not shown). Therefore, the conserved Leu-724 residue is likely required for Stat1 serine phosphorylation induced by cytokines as well as non-cytokine signaling pathways.

To further examine the Leu-724 mutations in a physiological background, human primary monocytes were transduced with lentiviral vectors containing Stat1WT, Stat1L724A, or Stat1L724I. Western blot analyses showed that high levels of exogenous Stat1 were expressed in the transduced cells (Fig. 2E, middle panel, lanes 3–8; and G) compared with the endogenous Stat1 expression in the primary monocytes (Fig. 2E, middle panel, lanes 1 and 2). In these primary cells, both Stat1 wild type and the L724I mutant were phosphorylated on Ser-727 (Fig. 2E, top panel, lanes 3, 4, 7, and 8; and F), whereas the L724A could not be phosphorylated on Ser-727 above the background level (Fig. 2E, top panel, lanes 5 and 6; and F). These results demonstrate that the two Leu-724 mutations also have a differential effect on Stat1S727 phosphorylation in physiological conditions.

Leu-724 Is Necessary for Stat1 Ser Phosphorylation by CaMKII in Vitro—Next, to further understand the mechanism for the impaired Ser-727 phosphorylation in the Stat1L724A mutant, we examined its ability for interaction with and phosphorylation by the multifunctional serine/threonine kinase CaMKII, which is responsible for IFN-γ-induced Ser-727 phosphorylation in Stat1 (29). GST fusion proteins containing the wild type Stat1 TAD, Stat1TADL724A, or Stat1TADL724I were prepared for in vitro binding assays and kinase assays. GST pull-down assays showed that the wild type Stat1 TAD could interact with CaMKII from nuclear extracts, whereas the Stat1TADL724A mutant was significantly impaired in this interaction (Fig. 3A, compare lanes 2 and 3). The Stat1L724I mutant could interact with CaMKII (Fig. 3A, lane 4) but at a lower level compared with the wild type. To further demonstrate that the lack of Ser-727 phosphorylation in Stat1L724A is a direct result of defective phosphorylation by the serine kinase, we performed in vitro kinase assays with purified CaMKII and GSTStat1TAD fusion proteins. The wild type Stat1TAD could be phosphorylated by CaMKII strongly (Fig. 3B, lane 5), and this phosphorylation has been shown to be specific on the Ser-727 residue (29). In contrast, the Stat1TAD containing L724A had little incorporation of 32P, whereas the Stat1TADL724I had a similar level of 32P incorporation compared with the wild type Stat1 TAD (Fig. 3B, lanes 6 and 7). Equal amounts of GST fusion proteins were used in these experiments (data not shown). Together, these results indicate that Leu-724 is required for the interaction between the Stat1TAD and its kinase CaMKII for Ser-727 phosphorylation of Stat1.

Recruitment of the Transcription Co-activator CBP/p300 to Stat1 Target Gene Promoters—The results shown above suggest that serine phosphorylation alone is not sufficient for Stat1 to activate transcription, as the Stat1L724I mutant has normal tyrosine and serine phosphorylation (Fig. 2) but still lacks transcription activity (Fig. 1B). To further understand the molecular basis of the defect in these mutants, we analyzed the assembly of Stat1 and the transcription co-activator CBP/p300 on the promoters of Stat1 target genes in the context of chromatin.

**FIGURE 3. Leu-724 is required for Ser-727 phosphorylation of Stat1 by CaMKII in vitro.** A, GST pull-down assays were performed with GSTStat1TAD fusion proteins and nuclear extracts from U3A cells. The bound proteins were analyzed by SDS-PAGE and Western blotting with an antibody specific for CaMKII. S1C, Stat1 TAD; WT, wild type; LA, L724A mutant; LI, L724I mutant. B, GST-fusion proteins containing wild type, L724A, or L724I Stat1TAD were used as substrates for in vitro CaMKII kinase assay. Incorporation of 32P into the Stat1TAD was visualized by autoradiography following SDS-PAGE. CaM, calmodulin.
Chromatin immunoprecipitation assays were performed on the Stat1 target genes IRF-1 (37) and GBP-1 (38) in stable cell lines containing the various Stat1 mutants. For the IRF-1 promoter, very little Stat1 was detected without IFN-γ/H9253 treatment (Fig. 4, top panel, lanes 4, 6, 8, 10, and 12). After treatment with IFN-γ/H9253 for 30 min, high levels of Stat1 were bound to the IRF-1 promoter, and none of the mutations in Stat1 affected the binding (Fig. 4; A, top panel; and B). CBP/p300 was not detected on the IRF-1 promoter in the untreated cells (Fig. 4, A, middle panel, lanes 4, 6, 8, 10, and 12). In cells containing wild type Stat1, the level of CBP/p300 on the IRF-1 promoter increased dramatically in response to IFN-γ/H9253 treatment (Fig. 4; A, middle panel, lane 3; and C). In contrast, the Stat1L724A, L724I, and S727A mutants had no detectable levels of CBP/p300 on the IRF-1 promoter (Fig. 4, middle panel, lanes 5, 7, and 9), indicating that both Leu-724 and Ser-727 are required for recruitment of CBP/p300 to the IRF-1 promoter. The Stat1S727D mutant could recruit a small amount of CBP/p300 (Fig. 4, middle panel, lane 11), and further quantitation analyses showed that it was ~20–30% of the amount of CBP/p300 recruited by the wild type Stat1 (Fig. 4C), suggesting that indeed negative charges play a role in the recruitment of CBP/p300. The results for the GBP-1 promoter are essentially similar to that of the IRF-1 promoter (Fig. 4, D–F). All together, these results indicate that both the Leu-724 and the Ser-727 are required for the recruitment of CBP/p300 to the STAT target gene promoters for cytokine-induced gene activation.

**Histone Acetylation at Stat1 Target Gene Promoters**—To further investigate the effect of these Stat1 mutants on chromatin remodeling at the IRF-1 and GBP-1 promoters, we analyzed the status of histone acetylation on the IRF-1 and GBP-1 promoters. ChIP assays with anti-acetyl-histone H4 showed that wild type Stat1 induced a significant increase in the level of acetylated-histone H4 (Fig. 5; A, lane 4; and B). Notably, mutations L724A, L724I, and S727A reduced the level of H4 acetylation to background levels (Fig. 5; A, lanes 6, 8, and 12; and B), whereas Stat1S727D had a residue level of H4 acetylation (Fig. 5; A, lanes 10; and B). Similarly, only wild type Stat1 could induce histone H4 acetylation on the GBP-1 promoter (Fig. 5, C and D). These results correspond to the effect of these mutants on the CBP/p300 recruitment shown in Fig. 4. All together, these results demonstrate that phospho-Ser-727 alone is not sufficient to recruit CBP/p300 and that both Leu-724 and Ser-727 are required for the recruitment of CBP/p300 to Stat1 target gene promoters in response to IFN-γ. 

**FIGURE 4. Recruitment of CBP/p300 to Stat1 target gene promoters.** Stable cell lines containing the various forms of Stat1 were either untreated or treated with IFN-γ for 30 min for IRF-1 and 1 h for GBP. ChIP assays were performed with anti-Stat1 and -CBP/p300. Genomic DNAs were isolated from the immunoprecipitation (IP) complex and input lysates for use as templates for PCR with primers specific for the IRF-1 (A) or the GBP-1 (D) promoters. The 32P-labeled PCR products were separated by electrophoresis and visualized by autoradiography. For each ChIP with a specific antibody, one representative result is shown from two to three independent experiments. Quantitation of the 32P-labeled PCR products for anti-Stat1 ChIP (B and E) and anti-p300 ChIP (C and F) were performed with a PhosphorImager (Amersham Biosciences). Results shown were relative intensities of the immunoprecipitation PCR products/input PCR products.
DISCUSSION

Protein phosphorylation is one of the most common mechanisms to regulate protein function. The critical regulatory steps of STAT functions such as nuclear translocation, DNA binding, and transcription activation are at the phosphorylation of two residues, one tyrosine and the other serine. The crystal structures of STAT proteins (17–19) showed that the members in the STAT family share considerable sequence and structural homology throughout the molecule, except the transcription activation domain, which has not been analyzed by crystallography. The LPMSP motif is the only sequence found to be conserved among the TADs of some of the STATs, which require serine phosphorylation for maximal transcription activity. Previous studies have shown that the serine residue is phosphorylated in response to ligand stimulation, and mutation of S727A impairs the transcription

FIGURE 5. Histone acetylation of Stat1 target gene promoters. Stable cell lines containing the various indicated Stat1 mutants were either untreated or treated with IFN-γ for 30 min for IRF-1 or 1 h for GBP-1. ChIP assays were performed with anti-acetyl histone H4 antibody for the IRF-1 (A) and the GBP-1 (C) promoters. The PCR products in Fig. 5, A and C, were quantitated by a PhosphorImager, and results are shown as ratios of immunoprecipitation (IP)/input for the IRF-1 (B) and the GBP-1 (D) promoters.

FIGURE 6. Regulation of Stat1-mediated transcription activation by Leu-724 and Ser-727. A schematic view of regulation of Ser-727 phosphorylation and CBP/p300 recruitment by the conserved residues Leu-724 and Ser-727 in Stat1 for transcription activation is shown. For ease of presentation, the Leu-724 and Ser-727 residues of only one molecule in the Stat1 dimer are depicted. To achieve optimal recruitment of CBP, we propose that both Leu-724 and phospho-Ser-727 were engaged in interaction with CBP as depicted for the wild type Stat1. For the Stat1L724I mutant, without the intact Leu-724, the interaction is not stable to achieve chromatin remodeling and transcription activation. Also omitted are other co-activators associated with Stat1 and CBP/p300. In addition, although we show tyrosine phosphorylation (PY) and serine phosphorylation (PS) in a sequential manner, serine phosphorylation can occur before or without tyrosine phosphorylation.
activity of Stat1 and Stat3 (20, 23–25). Our results using Leu-724 mutations demonstrate that, in addition to the phosphorylation of Ser-727, the conserved Leu-724 is also critical for the transcription activity of Stat1 (Figs. 1B and 6) and Stat3. Furthermore, these analyses indicate that Leu-724 is probably the controlling point for protein-protein interaction to recruit protein modification enzymes, such as CaMKII (serine kinase) and CBP/p300 (histone acetyl transferase), to regulate the transcription activity of the TADs of STAT proteins.

Several serine kinases, including CaMKII (29) and p38 mitogen-activated protein kinase (28), have been identified as the serine kinases for Ser-727 phosphorylation of Stat1 and extracellular signal-regulated kinase mitogen-activated protein kinase for Stat3 (26). However, it is not clear how specificity is determined between the STAT TADs and the serine kinases. Further analyses of the status of Ser-727 phosphorylation showed that the L724A mutation in Stat1 impaired Ser-727 phosphorylation in response to either IFN-γ (Fig. 2) or UV irradiation (data not shown). However, Stat1L724I can still be phosphorylated on Ser-727 in response to the respective stimulations. In the case of Stat3, the L724A mutation does not affect Ser-727 phosphorylation induced by interleukin-6 or OSM. It is possible that, because this motif contains two proline residues, it may have substantial structural flexibility as a recognition motif to recruit and present Ser-727 for phosphorylation by different kinases. Therefore, the control of STAT serine phosphorylation could be determined by type(s) of serine kinases activated in response to a particular stimulus.

We have suggested previously that the function of the phosphoserine is to enhance the interaction of STAT TAD with other proteins, and the negative charge is important (23) perhaps through engaging positively charged residues on the interacting proteins (39). The negatively charged residue Asp has been shown to mimic the effects of many enzymes that require auto-Ser/Thr phosphorylation with varying charged residue Asp has been shown to mimic the effects of many enzymes that require auto-Ser/Thr phosphorylation with varying.

Therefore, these structural and functional analyses of the STAT binding proteins could be further utilized to co-crystallize the STAT TAD with its binding partners for a complete understanding of STAT-mediated gene activation.

Acknowledgments—We thank George Stark and Ian Kerr for the U3A cell lines. We thank all of the laboratory members for their help and support.

REFERENCES

1. Levy, D. E., and Darnell, J. E., Jr. (2002) Nat. Rev. Mol. Cell Biol. 3, 651–662
2. O’Shea, J. J., Gadina, M., and Schreiber, R. D. (2002) Cell 109, (suppl.) S121–S131
3. Shuai, K., and Liu, B. (2003) Nat. Rev. Immunol. 3, 900–911
4. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
5. Askarian, M., Sedij, J. R., Yang, J., Jacobsen, N. G., Cebra, N., Yang, S. Y., Murphy, T. L., and Murphy, K. M. (2002) Nat. Immunol. 3, 549–557
6. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Cell 84, 443–450
7. Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7556–7561
8. Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenland, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. (1996) Cell 84, 431–441
9. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. M. (1994) Science 264, 1415–1421
10. Chivria, J. C., Kwock, R. P., Lamb, N., Hagwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859
11. Bhattacharya, S., Eckner, R., Grossman, S., Oldred, E., Arany, Z., D’Andrea, A., and Livingston, D. M. (1996) Nature 383, 344–347
12. Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E., Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15020–15026
13. Eckner, R., Even, M. E., Nozawa, S., Gersdes, M., DeCaprio, J. A., Lawrence, J. R., and Livingston, D. M. (1994) Genes Dev. 8, 869–884
14. Lu, B., Reichel, M., Fisher, D. A., Smith, J. F., and Rothman, P. (1997) J. Immunol. 159, 1255–1264
15. Morrigi, R., Berchtold, S., Friedrich, K., Standke, G. J., Kammer, W., Heim, M., Wissler, M., Stocklin, E., Gouilleux, F., and Groner, B. (1997) Mol. Cell. Biol. 17, 3663–3678
16. Paulson, M., Pisharodoy, S., Pan, L., Guadagno, S., Mai, A. L., and Levy, D. E. (1999) J. Biol. Chem. 274, 25343–25349
17. Becker, S., Groner, B., and Muller, C. W. (1998) Nature 394, 145–151
18. Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J. E., Jr., and Kuriyan, J. (1998) Cell 93, 827–839
19. Vinkemeier, U., Moarefi, I., Darnell, J. E., Jr., and Kuriyan, J. (1998) Science 279, 1048–1052
20. Weng, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
21. Kovarik, P., Mangold, M., Ramsauer, K., Heidari, H., Steinborn, R., Zetter, A., Levy, D. E., Muller, M., and Deckter, T. (2001) EMBO J. 20, 91–100
22. Morinobu, A., Gadina, M., Strober, W., Visconti, R., Fornace, A., Montagna, C., Feldman, G., Nishikomori, R., and O’Shea, J. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12281–12286
23. Zhang, J. J., Zhao, Y., Chait, B. T., Latham, W. W., Ritzi, M., Knippers, R., and Darnell, J. E., Jr. (1998) EMBO J. 17, 6963–6971
24. Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Muller, M., and Decker, T. (2003) Immunity 19, 793–802
25. Shen, Y., Schlissinger, K., Zhuo, X., Meffre, E. F., Quimby, F., Levy, D., and Darnell, J. E., Jr. (2004) Mol. Cell. Biol. 24, 407–419
26. Chung, J., Uchida, E., Grammer, T. C., and Blenis, J. (1997) Mol. Cell. Biol. 17, 6508–6516
27. Goh, K. C., Haque, S. J., and Williams, B. R. (1999) EMBO J. 18, 5601–5608
28. Kovarik, P., Stoiber, D., Eyers, P. A., Menghini, R., Neininger, A., Gaestel, M., Cohen, P., and Deckter, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13956–13961
29. Nair, J. S., Da Fonseca, C. J., Tjernberg, A., Sun, W., Darnell, J. E., Jr., Chait, B. T., and Zhang, J. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5971–5976
30. Nguyen, H., Ramana, C. V., Bayes, J., and Stark, G. R. (2001) J. Biol. Chem. 276, 33361–33368
31. Tassios, I., Xu, X., Hu, H., Kashyap, Y., Paik, P., Hu, Y., Lowell, C. A., and Iavashkov, M. (2004) Nat. Immunol. 5, 370–378
32. Yang, E., Herukskes, M. A., Schafer, O., Zakharova, N., and Darnell, J. E., Jr. (2000) J. Biol. Chem. 275, 13455–13462
33. Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. (1990) EMBO J. 9, 4477–4484
34. Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002) Science 295.

W. Sun, M. Snyder, and J. J. Zhang, unpublished results.
35. Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improtta, T., Darnell, J. E., Jr., Stark, G. R., and Kerr, I. M. (1993) *EMBO J.* 12, 4221–4228
36. Kovarik, P., Stoiber, D., Novy, M., and Decker, T. (1998) *EMBO J.* 17, 3660–3668
37. Yang, E., Lerner, L., Besser, D., and Darnell, J. E., Jr. (2003) *J. Biol. Chem.* 278, 15794–15799
38. Lew, D., Decker, T., Strehlow, I., and Darnell, J. E., Jr. (1991) *Mol. Cell. Biol.* 11, 182–191
39. DaFonseca, C. I., Shu, F., and Zhang, J. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 3034–3039
40. Fong, Y.-L., Taylor, W. L., Means, A. R., and Soderling, T. R. (1989) *J. Biol. Chem.* 264, 16759–16763
41. Pages, G., Brunet, A., L’Allemain, G., and Pouyssegur, J. (1994) *EMBO J.* 13, 3003–3010