Modification of the Stat1 SH2 Domain Broadly Improves Interferon Efficacy in Proportion to p300/CREB-binding Protein Coactivator Recruitment*

Received for publication, March 24, 2005, and in revised form, July 28, 2005 Published, JBC Papers in Press, August 17, 2005, DOI 10.1074/jbc.MS03263200

Yong Zhang†, Kazutaka Takami‡, Mindy S. Lo§, Guangming Huang†, Qing Yu†, William T. Roswit‡, and Michael J. Holtzman†§

From the †Department of Medicine, and ‡Department of Cell Biology, Washington University School of Medicine, St. Louis, Missouri 63110

A normal level of interferon (IFN) responsiveness via the Stat1 transcription factor is critical to the host, since decreased Stat1 signaling causes immune compromise and increased signaling is associated with inflammatory and neoplastic disease. Here we report how this balance may be influenced by novel alterations in the efficiency of Stat1 signaling. To enable disulfide-dependent and spontaneous formation of active Stat1 homodimer (as was done previously for Stat3), we engineered Stat1-CC with double-cysteine substitutions in the Src homology 2 (SH2)-homodimerization domain (at Ala-656 and Asn-658). In this case, however, mutant and wild-type Stat1 exhibited no difference in spontaneous dimerization. Moreover, Stat1-CC still required ligand-dependent Tyr-701 phosphorylation for function and exhibited hyperresponsiveness to IFN-β (that depends on Stat1/Stat2 heterodimerization) as well as IFN-γ (that depends on Stat1/Stat1 homodimerization). Hyperresponsiveness of Stat1-CC was accompanied by increased capacities for Tyr-701 phosphorylation and DNA binding, but these features were also found in a similarly substituted serine mutant (Stat1-SS) that showed no hyperresponsiveness to IFN-γ. This finding raised the possibility that SH2 domain mutations also influence downstream transcriptional efficiency. Indeed, each of these mutations also enhanced recruitment of the normally rate-limiting p300/CREB-binding Protein (CBP) coactivator to the transcriptional complex in proportion to the level of IFN-driven transactivation and gene expression. Additional modifications indicated that the mutant residues in the SH2 domain appeared to cooperate with Ser-727 in the C-terminal domain to regulate p300/CBP interaction with Stat1. The profile of IFN responsiveness translated into the same progressive increase in the level of viral clearance from Stat1- to Stat1-SS- to Stat1-CC-expressing cells. Thus, SH2 domain determinants may be modified to direct better Stat1 phosphorylation, DNA binding, and coactivator recruitment to fully improve IFN efficacy.

A complex and balanced program of IFN signal transduction is critical for host defense against pathogens as well as immune surveillance against cancer (1, 2). The protective actions of IFN rely on signaling through two IFN receptors (IFNAR for type I and IFNGR for type II IFNs, respectively) and the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway that includes receptor-associated JAK and STAT as well as downstream transcription factors, enhancers, and co-activators (3). For type I IFN (IFN-α/β), receptor-associated Jak1 and Tyk2 mediate the assembly of Stat1/Stat2 heterodimers that in concert with IRF-9 binds to ISRE promoter sites of IFN-α/β-responsive genes. For type II IFN (IFN-γ), receptor-associated Jak1 and Jak2 mediate formation of Stat1/Stat1 homodimers that bind to GAS sites of IFN-γ-responsive genes. For both STAT complexes, the subsequent recruitment of p300/CBP coactivators as well as other factors allows for transcription and consequent gene expression (4–7).

Despite the complexity in IFN signaling, the Stat1 transcription factor is remarkable as a critical functional component common to both type I and type II IFN signaling pathways. Indeed, the phenotype for viral infection of Stat1-null mice exhibits striking similarity to the one observed for loss of both type I and II IFN-receptor signaling (8–11). Susceptibility to viral infection is markedly increased in humans with mutations that inactivate Stat1 function (12, 13). By contrast, Stat1 activation (and Stat1-dependent gene expression) are detected in neoplasia and are invariably increased in asthmatic subjects (14–18). Analogous to the findings in neoplasia, Stat1 activation in asthma developed despite normal tissue levels of IFN and so was also labeled as constitutive. In both conditions, the teleological basis for altered Stat1 behavior under pathologic conditions is uncertain but may represent an attempt to improve immune surveillance. This possibility led us to question whether this signaling component of the immune system might be genetically engineered to better benefit the host compared with native Stat1 signaling.

The present experiments were therefore initiated to generate a Stat1 molecule with enhanced IFN responsiveness. In that context, we recognized that others have used crystal structures for Stat1, Stat3, and Stat4 as a guide (19–21) to engineer a recombinant Stat3 protein with double-cysteine substitutions to enable spontaneous homodimerization (by disulfide linkage) that allows ligand- and phosphorylation-independent DNA binding and transcriptional activity in transduced cell lines (22). This mechanism has been assumed in follow-up studies of Stat3 as well as Stat1 function (23–27). In the present study, however, we discover that double cysteine-substituted Stat1 (designated Stat1-CC) exhibited little constitutive activity or dimerization and was unexpectedly hyper-responsive to IFN-β as well as IFN-γ through a mechanism that in all cases still required ligand-dependent tyrosine phosphorylation. Com-

* This work was supported by grants from the National Institutes of Health (NHLBI), the Martin the Schaeffer Fund, and the Alan A. and Edith L. Wolff Charitable Trust. 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.

† To whom correspondence should be addressed: Washington University School of Medicine, Campus Box 8052, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-8970; Fax: 314-362-8987; E-mail: holtzmani@wustl.edu.

‡ The abbreviations used are: IFN, interferon; CBP, chromatin immunoprecipitation; EMVC, encephalomyocarditis virus; ICAM, intercellular adhesion molecule; Stat, signal transducer and activator of transcription, Tc-PTP, T cell protein-tyrosine phosphatase; CBP, CREB-binding protein; CREB, AMP-responsive element-binding protein; GFP, green fluorescent protein; MHC, major histocompatibility complex; mAb, monoclonal antibody; Ab, antibody; EM5A, electrophoretic mobility shift assay; ISRE, interferon-stimulated response element; FACS, fluorescence-activated cell sorter.
FIGURE 1. Design of retroviral vector constructs for Stat1, Stat1-SS, and Stat1-CC. Diagram illustrates the target sequence of site for double-serine and double-cysteine mutations in the SH2 domain of Stat1 and subsequent orientation into Stat1 and then into MSCV2.2 retroviral vectors expressing GFP with or without intervening IRES. For immunoprecipitation experiments, 3′×Flag sequence was added to generate Stat1-, Stat1-SS-, and Stat1-CC-Flag fusion genes. For dimerization experiments, Stat1- or Stat1-CC-Flag fusion sequence was inserted into Mx-neo retroviral vectors. The abbreviations for Stat1 domains are: NTD, N-terminus domain; CCD, coiled coil domain; DBD, DNA-binding domain; LD, linker domain; SH2D, SH2 domain; and TD, transactivation domain.

Above, in comparison to a double serine-substituted Stat1 (Stat1-SS) provided further insight into mechanism of action and focused attention at the level of transcriptional coactivator recruitment at a site, i.e. SH2 domain, that is distinct from previous studies of native or modified STAT function (22, 28–32). We also took advantage of our findings to broadly enhance IFN signaling capacity in this natural setting as well.

MATERIALS AND METHODS

Retroviral Vector Construction and Transfection—Human Stat1-CC cDNA with Ala-656 to Cys-656 and Asn-658 to Cys-658 substitutions was generated using 5′-CGCAATTTCAAGTCTGCTTCTGAG-TGTATTTCTGAGAATCCTCCTGAAG-3′ as upstream and 5′-CTTCAGGGATTTCTCAGGAATACACTCAACAGCATGACTT-GTAATTGCG-3′ as downstream primers in the QuikChange site-directed mutagenesis kit (Stratagene). Stat1-SS cDNA with Ala-656 to Ser-656 and Asn-668 to Ser-668 substitutions was generated using 5′-CGCAATTACAAAGTCTGCTTCTGAG-TGTATTTCTGAGAATCCTCCTGAAG-3′ as upstream and 5′-CTTCAGGGATTTCTCAGGAATACACTCAACAGCATGACTT-GTAATTGCG-3′ as downstream primers. Full-length Stat1, Stat1-SS, or Stat1-CC with or without C-terminal 3×Flag reporter sequence was used as the corresponding double mutants with Ser-727 to Ala-727 substitution were inserted into retroviral vector MSCV2.2 (obtained from K. M. Murphy, Washington University) or Mx-neo (obtained from T. Kitamura, University of Tokyo) to generate the following vectors: Stat1-IRES-GFP, Stat1-Flag-IRES-GFP, and Stat1(S727A)-Flag-IRES-GFP, Stat1-SS-IRES-GFP, Stat1-SS-Flag-IRES-GFP, and Stat1(S727A)-SS-Flag-IRES-GFP, and Stat1-CC-IRES-GFP, Stat1-CC-Flag-IRES-GFP, and Stat1(S727A)-CC-Flag-IRES-GFP or Mx-Stat1-Flag-neo and Mx-Stat1-CC-Flag-neo (Fig. 1). For transactivation and co-immunoprecipitation assays, we also generated Stat1, Stat1-SS, or Stat1-CC cDNA with C-terminal GFP sequence to generate Stat1-GFP, Stat1-SS-GFP, and Stat1-CC-GFP vectors. A cDNA fragment for Stat1 and Stat1-CC encoding a Tyr-701 to Phe-701 substitution was similarly used to generate vectors encoding Stat1(Y701F)-GFP or Stat1(Y701F)-CC-GFP, and these vectors along with Stat1- and Stat1-CC-GFP vectors were used to test the requirement for Tyr-701 phosphorylation. Stat1-deficient U3A and Type I IFNAR2-deficient U5A cells were obtained from G. Stark (Cleveland Clinic). The Phoenix-Ampho packaging cell line was obtained from G. Nolan (Stanford University) and transfected as described previously (33). Transduced cells were sorted using a FACSVantage SE to isolate a population of cells that were >90% GFP-expressing. To generate cells expressing Stat1-GFP and Stat1-Flag or Stat1-CC-GFP and Stat1-CC-Flag, we transfected Mx-Stat1-Flag-neo or Mx-Stat1-CC-Flag-neo into MSCV-Stat1-GFP or MSCV-Stat1-CC-GFP expressing U3A cells, selected transduced cells in G418, and verified similar expression levels by Western blotting for Flag and GFP.

Analysis of Stat1-dependent Gene Expression—For flow cytometry, samples of 1×10⁵ cells were treated with or without recombinant human IFN-γ (Genentech) or IFN-β (PBL Biomedical Laboratories) and analyzed for expression of ICAM-1 or Class I major histocompatibility complex (MHC) using phycoerythrin-conjugated mouse anti-human ICAM-1 mAb 84H10 or HLA-A,B,C mAb from Pharmingen and a FACSscan interfaced to CellQuest software (BD Biosciences). For real-time PCR, target mRNA levels were quantified with 5′-GGAGGGAGAATTTACCTGAGGACAT-3′ and 5′-CTTCCCTACCTCCAGTTTGT-3′ for IRF-1 or 5′-CTTACCTGATGAGAATGAGAATGAG-3′ and 5′-GTTTCAGGGATGTGAGCAGCTACTC-3′ for GBP as forward and reverse primers in the TaqMan One-Step system (PE Biosystems) with glyceraldehyde-3-phosphate dehydrogenase (nucleotides 37–910) serving as a control.

Analysis of Stat1 Activation and Dimerization—For Western blotting, cells were lysed as described previously (34). polyvinylidene difluoride membranes were blotted with rabbit anti-human phospho-Stat1 (Tyr-701) Ab and rabbit anti-mouse phospho-Stat3 (Tyr-705) from Cell Signaling Technology, rabbit anti-mouse phospho-Stat2 (Tyr-689) Ab from Upstate Cell Signaling Solutions, mouse anti-human Stat1 (N terminus) mAb from BD Biosciences, rabbit anti-human Stat2 (C terminus) Ab, and rabbit anti-human Stat3 from Santa Cruz Biotechnology, mouse anti-FLAG Ab from Sigma, and mouse anti-GFP Ab from Roche Applied Science. For phosphatase sensitivity assays, cell lysates were prepared in buffer without sodium orthovanadate, sodium pyrophosphate, or sodium fluoride and then incubated with the 45-kDa splice variant of T cell protein-tyrosine phosphatase (Tc-PTP; New England BioLabs) for 1 h at 30 °C. For Stat1 co-immunoprecipitation, cell lysates were incubated with agarose beads conjugated to anti-Stat1 Ab (10 μg, Santa Cruz Biotechnology), and for dimerization detection, cell lysates were incubated with anti-GFP Ab or anti-FLAG Ab followed by protein G-Sepharose (Amersham Biosciences). For electrophoretic mobility shift assay (EMSA), whole cell lysates or nuclear extracts were incubated with 32P-labeled double-stranded DNA oligonucleotide (1 ng) containing the GAS motif from human ICAM-1 gene promoter, the ISRE motif from ISG15 gene promoter, or the GAS motif from the M67-SIE probe as described previously (7, 35, 36). For supershifts, anti-Stat1 or anti-Stat3 mAb (1 μg) was added to the reaction.

Analysis of Stat1-Coactivator Interaction—For luciferase reporter gene transactivation assays, Stat1-1GFP, Stat1-SS-GFP, or Stat1-CC-GFP in MSCV2.2 (0.5 μg) was co-transfected with pGAS-Luciferase or pISRE-Luciferase plasmid (1 μg, Stratagene) into U3A cells using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. After 24 h, transfected cells were treated with IFN-γ (100 units/ml) or IFN-β (1000 units/ml) for 12 h and then split into aliquots

Stat1 and IFN Responsiveness
Stat1 and IFN Responsiveness

for analysis of luciferase activity according to the manufacturer’s instructions (Promega) and GFP level by flow cytometry. GFP level was used to correct for transfection efficiency. For chromatin immunoprecipitation (ChIP) assays, Stat1-Flag-IRES-GFP, Stat1-SS-Flag-IRES-GFP, or Stat1-CC-Flag-IRES-GFP-expressing U3A cells were treated with IFN-γ or IFN-β for 30 min at 37 °C (1 × 10^6 cells/condition), incubated with 1% formaldehyde in phosphate-buffered saline for 10 min at 25 °C to cross-link protein-DNA and protein-protein complexes. The cell lysates were sonicated to yield DNA fragments from 200–1000 bp, debris was removed by centrifugation, and the supernatant was diluted 1:10 (v/v). An aliquot was kept as a loading control for input/sample. Remaining supernatant was incubated with anti-Stat1 or control mouse IgG-agarose beads (5 μL, Santa Cruz Biotechnology) and salmon sperm DNA (20 μg) for 16-h rotation at 4 °C. Immunoprecipitated DNA was extracted with phenol/chloroform and ethanol precipitation, and a 10% aliquot was analyzed by real-time PCR using primers for human IFN-1 promoter (forward and reverse primer pairs: 5'-CTTCGCCGCTAGCTCTAC-3' and 5'-CCCATTGCGCGCTGCTG-3') that binds Stat1/Stat1 and Stat1/Stat2 dimers (37). For protein-protein interaction with ChIP, cell lysates were sheared using a 29-gauge syringe instead of sonication and incubated with agarose beads conjugated to anti-Flag Ab (20 μg, Sigma) or anti-p300 Ab (5 μg, Santa Cruz Biotechnology), washed with immunoprecipitation buffer, and analyzed by Western blotting with anti-Stat1, anti-phospho-Stat1, anti-Stat2, anti-phospho-Stat2 Ab, or anti-p300 Ab as described above.

Viral Inoculation and Monitoring—Mouse encephalomyocarditis virus (EMCV, VR-129B) was obtained from ATCC and used to inoculate cell cultures as described previously (38). Real-time PCR for EMCV was performed using 5'-CTGCCCTGGTTCCGC-3' and 5'-TGGCTGACATCAAAGTTGGAG-3' as forward and reverse primers, respectively.

Flow cytometry to monitor cell viability was performed using propidium iodide staining as described previously (39).

RESULTS

Stat1 Hyperresponsiveness to Type I and II IFN—We first generated a Stat1 mutant (Stat1-CC) with double-cysteine substitutions at sites (Ala-656 and Asn-658) where the C-terminal domains of the Stat1 homodimer are in close proximity. This mutation was expected to allow for spontaneous disulfide bonding and Stat1 homodimerization as described for Stat3 (22). Therefore, we also generated a Stat1 mutant (Stat1-SS) with homologous serine substitutions to confer similar steric effects but no capacity for possible disulfide bond formation. Functional activity of wild-type and mutant Stat1 proteins were compared after stable expression in Stat1-deficient U3A cells, where mutant Stat1 was expressed similarly to wild type. In contrast to predictions, we found no spontaneous expression of IFN-γ-responsive genes in Stat1-CC-expressing cells (as exemplified by unchanged basal ICAM-1 gene expression in Fig. 2) but instead found increased responsiveness to IFN-γ in Stat1-CC- versus Stat1-SS- or Stat1-expressing cells (exemplified by increased IFN-γ-induction of ICAM-1 and MHC Class I gene expression in Fig. 2). In addition, we found that Stat1-CC conferred increased responsiveness of IFN-β-inducible genes at baseline and after IFN-β treatment (as exemplified by basal and IFN-β-inducible MHC Class I gene expression in Fig. 2). Unexpectedly, hyperresponsiveness of IFN-β-inducible genes was also present in Stat1-SS-expressing cells, although at an intermediate level between Stat1- and Stat1-CC-expressing cells. As developed below, each of these findings suggested an alternative mechanism to the one proposed for spontaneous disulfide-dependent dimerization of Stat3.

Mutant Stat1 Requirement for Activation—Although we detected no constitutive expression of IFN-γ-responsive genes (e.g. ICAM-1), we did find low level induction of IFN-β-responsive genes (e.g. MHC Class I) even without IFN treatment. To determine whether this basal activity might be due to spontaneous dimerization of Stat1-CC (allowing for signaling without phosphorylation), we next generated a Stat1-CC protein with mutation of the Tyr-701 that is required for ligand-dependent dimerization of Stat1. This Stat1-CC-Y701F mutant, unlike Stat1 or Stat1-CC, did not respond to IFN-γ or IFN-β stimulation (Fig. 3a,b). In addition, the increased basal level of MHC Class I gene expression observed with Stat1-CC transduction was completely abolished by the introduction of this mutation (Fig. 3b). This requirement for Tyr-701 phosphorylation suggested to us that low level Stat1-CC activity at baseline might be due to small amounts of constitutive production of IFN-β. In accordance with this hypothesis, the increased basal expression activity was also lost when Stat1-CC was expressed in IFNAR-deficient U5A cells that are unable to respond to IFN-β (Fig. 3c). Thus, the low level of Stat1-CC action under baseline conditions appears to depend on sim-
ilarly low levels of endogenous type I IFN release under control conditions. Indeed, under the present cell culture conditions, we found low basal levels of IFN-β mRNA that did not vary significantly with retroviral transduction but were inducible with viral infection (data not shown). The capacity of Stat1-CC to enhance IFN-β-dependent signaling thereby explained the basal activity in Stat1-CC-expressing cells as being driven by constitutive release of IFN-β.

Mutant Stat1 Capacities for Dimerization, Tyrosine Phosphorylation, and DNA Binding—In view of the apparent absence of spontaneous function for mutant Stat1, we next conducted experiments to more directly monitor dimerization of wild-type and mutant Stat1 proteins. Accordingly, we labeled both wild-type and mutant Stat1 with distinct tags (GFP or Flag) to monitor the capacity of each Stat1 species for dimerization. In the case of spontaneous dimerization, we found no difference in behavior between wild-type Stat1 and Stat1-CC (Fig. 4). For each species of Stat1, we detected a low level of spontaneous dimerization that is likely mediated by N-domain dependent interactions (40, 41) and so is apparently unaffected by the present mutation in the SH2 domain. By contrast, in the case of IFN-γ stimulation, we detected an increase in Stat1-CC compared with wild-type Stat1 dimerization (Fig. 4). This finding is again consistent with increases in IFN-inducible gene expression for mutant versus wild-type Stat1.
Based on the requirement for Tyr-701 phosphorylation for mutant Stat1 function, we next assessed the phosphorylation-dephosphorylation behavior of wild-type and mutant Stat1 proteins. Assessments of nuclear retention, tyrosine phosphorylation, and DNA binding each provided evidence of prolonged activation for Stat1-CC and Stat1-SS in comparison to wild-type Stat1. Thus, Stat1 and Stat1-CC displayed similar localization to the cytoplasmic compartment under baseline conditions (without IFN treatment), but Stat1-CC was retained in the nucleus significantly longer than Stat1 in response to IFN-β/H9253 or IFN-β/H9252 (data not shown). Nuclear retention was accompanied by prolonged Tyr-701 phosphorylation of mutant relative to wild-type Stat1 in response to IFN-β/H9253 or IFN-β/H9252 (Fig. 5a). Prolonged Tyr-701 phosphorylation was likely based on resistance to Stat1 dephosphorylation, since initial phosphorylation levels were similar for wild-type and mutant Stat1, and any later increase in tyrosine phosphorylation was blocked by treatment with a tyrosine kinase inhibitor. This possibility is further developed below by testing sensitivity to phosphatase treatment.

In concert with prolonged Tyr-701 phosphorylation, we also found increased and prolonged DNA binding of each mutant Stat1 compared with wild-type Stat1 proteins (Fig. 5b). However, neither increased nuclear retention, Tyr-701 phosphorylation, nor DNA-binding was sufficient to fully explain increased function of the present Stat1 mutations. Thus, the level of Stat1-SS DNA binding is increased after IFN-γ, but Stat1-SS exhibits no increase in IFN-γ-responsiveness; in contrast, the level of Stat1-SS DNA binding is only marginally increased after IFN-β, but Stat1-SS exhibits a significant increase in IFN-β-responsiveness. These findings indicated that Stat1 DNA-binding was dissociated from transcriptional activity under some circumstances and so prompted further experiments aimed at defining additional mechanisms for mutant Stat1 hyperresponsiveness to IFN.

To better define the mechanisms for IFN responsiveness, we considered whether Stat1-CC exhibits higher affinity for Stat2 or how it might otherwise protect Stat2 from dephosphorylation. We first found that Stat2 affinity appeared similar for Stat1, Stat1-SS, and Stat1-CC, since the levels of IFN-γ-inducible interaction were similar at early times (0.3 h) before dephosphorylation becomes significant (Fig. 6a). To examine protection from dephosphorylation, we monitored wild-type and mutant Stat1 behavior after treatment with the Stat1-active protein-tyrosine phosphatase TC-PTP. This experiment showed that Stat1-CC exhibited resistance to TC-PTP-mediated dephosphorylation after IFN-β or IFN-γ treatment (Fig. 6b and data not shown). In contrast, Stat2 was equally susceptible to dephosphorylation when coupled with wild-type or mutant Stat1. (Fig. 6b). These data suggest that Stat1-CC resistance to dephosphorylation and consequent preservation of the
Stat1/Stat2 dimer may protect Stat2 from dephosphorylation after IFN-β, while preservation of the Stat1/Stat1 dimer may preserve phosphorylation after IFN-γ treatment.

We also considered whether other STAT proteins may form heterodimers with Stat1 as has been described for Stat3 (36). Under the present conditions in U3A cells, Stat3 expression is decreased compared with Stat1 (Fig. 6c), consistent with previous reports by others (36, 42). Nonetheless, the low level of detectable Stat3 phosphorylation and heterodimer formation is no different in wild-type Stat1-, Stat1-SS-, and Stat1-CC-expressing cells (Fig. 6, a and c). Similarly, DNA binding to the M67-SIE GAS probe after IFN-γ stimulation indicated that wild-type Stat1- and Stat1-CC-expressing cells form the same, single DNA-protein complex after IFN-γ or IFN-β treatment, and this complex is supershifted by anti-Stat1 Ab (Fig. 6d). This result is similar to findings by others (43) and stands in contrast to the development of Stat1/Stat1-, Stat1/Stat3-, and Stat3/Stat3-DNA binding that is detectable in HepG2 cells that have higher Stat3 levels (36) and (data not shown). Thus, the relatively low level of Stat3 phosphorylation in U3A cells does not translate into significant DNA binding and is therefore unlikely to contribute to the change in Stat1-CC function that we observe.

Mutant Stat1 and p300/CBP Recruitment—To this point, our experiments provided evidence of increases in IFN-inducible Tyr-701 phosphorylation, nuclear retention, and DNA binding for mutant Stat1, but as noted above, these alterations in mutant Stat1 behavior were still insufficient to fully account for increased function of the present Stat1 mutations. Because levels of DNA binding by EMSA did not fully correlate with function, we next aimed at further analysis of downstream transcriptional activities for wild-type versus mutant Stat1 proteins. For these experiments, the available tools prompted us to focus on IRF-1 gene expression. The IRF-1 gene also exhibits IFN-γ induction of IRF-1 mRNA that is similar for Stat1 and Stat1-SS but increased for Stat1-CC, and IFN-β induction is intermediate for Stat1-SS and highest for Stat1-CC (Fig. 7). The profile is therefore similar to the one that we found for ICAM-1 and MHC Class I gene expression and allowed for
Stat1 and IFN Responsiveness

additional experiments aimed at the mechanism for enhanced function of mutant Stat1.

Using a luciferase-reporter assay for gene transcription, we found that IFN-γ- and IFN-β-induced transcription of GAS- and ISRE-containing reporter plasmids, respectively, were closely correlated with previously observed functional capacities of Stat1, Stat1-SS, and Stat1-CC for driving endogenous gene expression (Fig. 8a). In this assay, we also confirmed a requirement for Tyr-701 phosphorylation to achieve any responsiveness of wild-type or mutant Stat1 to either type of IFN. Because gel-shift assays may not correlate precisely with Stat1 DNA binding in situ, we next assessed the level of endogenous wild-type and mutant Stat1 binding to native promoter elements using a ChIP assay. Using anti-Stat1 Ab and PCR-based quantification of immunoprecipitated IRF-1 promoter sequence bound to wild-type or mutant Stat1, we found increases in mutant Stat1 promoter binding that correlated closely with previous results from EMSA. In particular, results from the ChIP assay also indicated that Stat1-SS exhibited increased binding to the promoter (but no increased function) after IFN-γ and no significant increase in DNA-binding (despite markedly increased function) after IFN-β treatment (Fig. 8b).

Since mutant Stat1 binding to the gene promoter did not by itself predict transcriptional function, we hypothesized that transcription complexes containing the mutant Stat1 proteins were also more effective for transcriptosome assembly. Because p300/CBP is rate-limiting for Stat1-dependent transcription and appears to bind to the Stat1 transactivation domain near the site of the introduced Stat1 mutations (4, 5, 32, 44), we next determined whether the modified Stat1 proteins were better able to recruit p300/CBP coactivator. For these experiments, we used the same protocol for DNA-protein and protein-protein crosslinking and the same wild-type and mutant Stat1-Flag vectors as for the ChIP assay, followed by immunoprecipitation with anti-Flag or anti-p300 antibodies and Western blotting. We proceeded to ChIP assay after we were unable to co-immunoprecipitate Stat1 and p300/ CBP using anti-Stat1 or anti-p300 Ab. We note that most reports use GST-fusion proteins to show direct Stat1-p300/CBP interaction (4, 45). Others have also failed to detect direct interaction with anti-Stat1 Ab and used biotinylated GAS-oligonucleotides and streptavidin-agarose beads to show Stat1-p300/CBP interaction (46). Thus, the need for the ChIP assay suggests a requirement for DNA in the interaction of the endogenous proteins, but other technical issues (such as blocked antibody binding sites) cannot be fully excluded.

Using this strategy, we were able to demonstrate a profile of p300 interaction with wild-type and mutant Stat1 proteins that correlated precisely with transactivating function. Thus, Stat1-CC exhibited increased p300 binding after both IFN-γ and IFN-β treatment, while Stat1-SS exhibited increased p300 binding only after IFN-β treatment (Fig. 8c). The increase in mutant Stat1-p300 binding was also detectable with anti-phospho-Stat2 Ab after IFN-β treatment, indicating that the Stat1/Stat2 heterodimer was also capable of enhanced p300 binding (Fig. 8d). These findings, in conjunction with gene expression and transcriptional activity assays, implied that mutant Stat1 increased IFN-responsiveness by enhancing the level of Stat1 interaction with p300/CBP at the site of gene transcription, rather than solely by changing the level of tyrosine phosphorylation or DNA binding.

Previous work on Stat1-p300/CBP interaction had indicated that the Stat1 C-terminal domain (via the Ser-727 phosphorylation site) controlled this interaction during stimulation with IFN-γ (32). We also found that Ser-727 mutation caused a decrease in native Stat1 interaction with p300/CBP and consequent gene expression after IFN-γ and detected a similar decrease after IFN-β stimulation (Fig. 9, a and b).

These inhibitory effects of Ser-727 mutation were also detected for Stat1-SS and Stat1-CC and were often more easily detected in this case since p300/CBP recruitment and gene expression were present at higher levels (Fig. 9, a and b). Stat1-SS and Stat1-CC increases in p300/CBP recruitment and gene expression persisted despite the loss of Ser-727 suggesting that the distal SH2 domain (at Cys/Ser-656 and -658) likely cooperates with the proximal C-terminal domain (at Ser-727) to regulate p300/CBP interaction with Stat1-CC-CBP and Stat1-SS. We also note that Stat1-SS can appear to exhibit increased binding to p300/CBP if assessed with anti-phospho-Stat1 Ab, since Stat1-SS is relatively resistant to dephosphorylation that occurs during the multi-step ChIP assay (Fig. 9a, lane 5 versus lane 1). However, Stat1-SS interaction with p300/CBP is no different from wild-type Stat1 if assessed with the anti-Stat1 Ab that is not influenced by this variation.

Enhanced Viral Clearance in Cells Expressing Modified Stat1—Resistance to viral infection often relies on both IFN-α/β and IFN-γ signaling (12, 13). We therefore predicted that the dual action of Stat1-CC or Stat1-SS for boosting responses to both types of IFN would be especially useful in also boosting antiviral defense. Indeed, we next found that enhanced IFN efficacy translated into improved antiviral action in Stat1-CC and Stat1-SS versus Stat1-expressing or Stat1-null U3A cells infected with EMCV (Fig. 10a). In general, the capacity of modified Stat1 to improve viral clearance correlated with its capacity to increase IFN responsiveness. The improvement in viral clearance could simply reflect an increase in host cell death rates, and indeed, we observed more cell death after IFN treatment. However, in the case of EMCV infection, Stat1-CC expression actually conferred net protection from virus-in-
duced cell death (Fig. 10). Taken together, these results indicate that the improvement in viral clearance mediated by Stat1-CC or Stat1-SS was due at least in part to inhibition of viral replication and consequent prevention of viral cytopathic effect.

**DISCUSSION**

The present study demonstrates that specific modification of the Stat1 SH2 domain can markedly increase responsiveness to both type I and II IFN and defines the mechanism for this action. The findings have biochemical and practical implications for IFN signaling that have not been recognized previously, and each will be discussed separately.

From a biochemical standpoint, we show that mutagenesis of Stat1 SH2 domain (at Ala-656 and Asn-658) has several unexpected functional consequences: (i) preservation of ligand-dependent activation, (ii) increased capacity for Tyr-701 phosphorylation and DNA binding, and (iii) enhanced recruitment of p300/CBP coactivator to the transcriptional complex. These actions were unexpected because similar mutagenesis (i.e. double-cysteine substitution) of Stat3 was reported to result in spontaneous, ligand-independent homodimerization due to disulfide bond formation between the introduced cysteine residues (22). In the present case, however, we found that a similarly substituted Stat1 molecule exhibited activity only after stimulation with ligand (IFN-α or IFN-γ), since activity was lost if IFN signaling was disrupted either by loss of the IFN receptor or mutation of Tyr-701. Moreover, Stat1-CC exhibited no increase in spontaneous dimerization compared with wild-type Stat1. In addition, we recognized that disulfide-linked Stat1/Stat1 homodimerization was unlikely to explain the broader action of Stat1-CC on IFNα/β signaling through the Stat1/Stat2 heterodimer...
Stat1 and IFN Responsiveness

and of course could not account for the functionality of a similarly substituted serine mutant Stat1-SS.

Indeed, our insights into Stat1-CC mechanism were derived from further analysis of Stat1-CC behavior and comparison to Stat1-SS. Thus, Stat1-CC exhibited increased prolonged nuclear translocation, Tyr-701 phosphorylation, and DNA binding in response to IFN-γ and IFN-β, and each of these characteristics could be linked to one another and to increased transactivating function. However, Stat1-SS exhibited each of these same characteristics but was unable to alter IFN-γ responsiveness. This discrepancy in capacities for DNA-binding and transcriptional activation led to further study of transcriptional mechanism and ultimately close correlation of functional activity with the capacity of each mutant to recruit the p300/CBP coactivator of gene transcription. The level of p300/CBP recruitment to the transcriptional complex is rate-limiting for Stat1-dependent transcription, and in the past, this function appeared to depend on p300/CBP CH3 domain interaction with Stat1 C-terminal transactivation domain (4, 5, 32, 44). Thus, an improvement in p300/CBP recruitment for mutant Stat1 represents a suitable candidate mechanism for increases in native or mutant Stat1 transactivating function. The mutant portion of the SH2 domain is near the C terminus and so is likely exposed to the C-terminal region in space. Therefore, it is perhaps not surprising that this SH2 site could function in concert with the C terminus to influence p300/CBP recruitment. Based on the proximity of the present SH2 domain mutation sites to the C-terminal Ser-727, it is reasonable that this region cooperates in p300/CBP interaction with Stat1. In support of this possibility, it appears that p300/CBP may also bind near this region to achieve lysine acetylation in Stat3, and a similar motif (including Lys-679) exists in Stat1 (47). In the case of Stat3, p300/CBP interaction may aid in dimerization, while the present results for Stat1 highlight a downstream role in transcriptional activation. The present structural mechanism for Stat1-p300/CBP interaction and the consequences for Stat1 transcriptional assembly are diagrammed in Fig. 11.

In that context, we note that definition of p300/CBP interaction with Stat2 or the Stat1/Stat2/IRF-9 (ISGF3) complex has been less well defined. Initial study indicated interaction between the CH1 domain of p300/CBP and the Stat2 C terminus (6) as well as an influence of Stat1 (via Ser-727) on IFN-β responsiveness (48), but our work is the first to up-regulate p300/CBP interaction with ISGF3 by modifying its components. Since Stat1-SS was more effective for p300/CBP recruitment after IFN-β compared with IFN-γ, it is likely that there are distinct and perhaps synergistic determinants for ISGF3-versus Stat1-mediated recruitment of p300/CBP. In both cases, however, we find that the SH2 domain modification improves p300/CBP recruitment over the wild-type Stat1. Thus, the IFN signaling system may be naturally adjusted in a manner that does not permit maximal p300/CBP recruitment. Presumably, this maximal setting would lead to detrimental effects on the host in the long term but may nonetheless be advantageous for the acute host response (e.g. infection) or in other special settings (e.g. IFN treatment).

Our findings thereby raise practical considerations for improving IFN efficacy. In particular, we provide a rational basis for targeting Stat1 capacities for both transcriptional efficiency as well as DNA binding properties in any effort to enhance IFN responsiveness. Prior attempts to improve IFN actions have ranged from delivery of IFN itself to modification of IFN signaling, but each has had inherent drawbacks. Overexpression or direct administration of IFN has been limited for treatment of infectious or neoplastic disease because pathogens exhibit variable susceptibility to Type 1 versus Type 2 IFN and IFN therapy is associated with significant toxicities. Modification of IFN signaling can be approached by inhibition of endogenous inhibitors of Stat1 phosphorylation (e.g. SOCS1) or DNA binding (e.g. PIAS1), but each is less specific than Stat1 for influencing IFN-responsiveness (49, 50). Thus, the ability to selectively yet broadly boost IFN efficacy by enhancing Stat1 function could be an advantageous approach for further therapeutic development. Indeed, increased levels of Stat1 activation in response to normal levels of IFN may also be found naturally in inflammatory diseases such as asthma (16), suggesting that the host may be evoking Stat1 signaling capacity for improved anti-viral response in this setting as well. In this case, however, the balance between Stat1-dependent inflammation and immunity may not yet be optimized to obtain the same benefit as a targeted mutagenesis approach. Indeed, the present results indicate that IFN efficacy for viral clearance and targeted cell death may be improved under the direction of modified Stat1 activity.

Acknowledgments—We thank Yael Alevy, Eugene Agapov, and Douglas Dean for expert advice and assistance.

REFERENCES

1. Sen, G. C. (2001) Annu. Rev. Microbiol. 55, 255–281
2. Dunn, G. P., Old, I. J., and Schreiber, R. D. (2004) Immunity 21, 137–148
3. Levy, D. E., and Darnell, J. E., Jr. (2002) Nat. Rev. Mol. Cell. Biol. 3, 651–662
4. Zhang, J. J., Vinkemeier, U., Gu, W., Chaturvedi, D., Horvath, C. M., and Darnell, J. E., Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15092–15096
5. Hovrui, A. E., Xu, L., Korzus, E., Brad, G., Kalafus, D., Mullen, T. M., Rose, D. W., Rosenfeld, M. G., and Glass, C. K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1074–1079
6. Bhattacharya, S., Ecker, N., Grossman, S., Oldread, E., Arany, Z., D’Andrea, A., and Livingston, D. M. (1996) Nature 383, 344–347
7. Look, D. C., Roswit, W. T., Frick, A. G., Gris-Aley, Y., Dickhaus, D. M., Walter, M. J., and Holtzman, M. I. (1998) Immunity 9, 871–880
8. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Cell 84, 443–450
9. Park, C., Li, S., Cha, E., and Schindler, C. (2000) Immunity 13, 795–804
10. Shimoda, K., Kato, K., Aoki, K., Matsuda, T., Miyamoto, A., Shibamori, M., Yasamashita, M., Numata, A., Takase, K., Kobayashi, S., Shibata, S., Asano, Y., Grono, H., Sekiguchi, K., Nakayama, K., Nakayama, T., Okamura, T., Koamura, S., Nihou, Y., and Nakayama, K. (2000) Immunity 13, 561–571
11. Durbin, J. E., Fernandez-Sesma, A., Lee, C.-K., Sharma, T., Frey, A. B., Moran, T. M., Yukonanovic, S., Garcia-Sastre, A., and Levy, D. E. (2000) Immunity 16, 4220–4228
12. Dupuis, S., Dargemont, C., Fieschi, C., Thomassin, N., Rosei, M., Harris, J., Holland, S. M., Schreiber, R. D., and Casanova, J.-L. (2001) Science 293, 300–303
13. Dupuis, S., Jouanguy, E., Al-Hajjar, S., Fieschi, C., Al-Mohsen, I. Z., Al-Jumah, S., Yang, K., Chappier, A., Edenschenk, C., Eid, P., Ghonaima, A. A., Tufenkeji, H., Frayha, H., Al-Gazlan, S., Al-Rayes, H., Schreiber, R. D., Greener, I., and Casanova, J.-L. (2003) Nat. Genet. 33, 388–393
14. Zong, C., Van, B., August, A., Darnell, J. E., Jr., and Hanafusa, H. (1996) EMBO J. 15, 4515–4525
15. Frank, D. A., Mahajan, S., and Ritz, K. (1997) J. Clin. Invest. 100, 3140–3148
16. Sampath, D., Castro, M., Look, D. C., and Holtzman, M. J. (1999) J. Clin. Invest. 103, 1353–1361
17. Guo, F. H., Comhair, S. A., Zheng, S., Dweik, R. A., Eissa, N. T., Thomasen, M. J., Calhoun, W., and Eruruzum, S. C. (2000) J. Immunol. 164, 5970–5980
18. Bromberg, J. (2002) J. Clin. Invest. 109, 1139–1142
19. Becker, S., Groner, B., and Muller, C. W. (1998) Nature 394, 145–151
20. Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J. E., Jr., and Kuriyan, J. (1998) Cell 93, 827–839
21. Chen, X., Bhandari, R., Vinkemeier, U., van den Akker, F., Darnell, J. E., Jr., and Kuriyan, J. (2003) Protein Sci. 12, 361–365
22. Bromberg, J. F., Wrzeszczynska, M. H., Zhao, Y. H., Pestel, R. G., Alasoo, K., Gabrilovich, D., Heller, R., Coppola, D., Dalton, W., Jove, R., Pardoll, D., and Yu, H. (2004) Nat. Med. 10, 48–54
23. Inoue, H., Ogawa, W., Ozaki, M., Haga, S., Matsumoto, M., Furukawa, K., Hashimoto, N., Kido, Y., Morii, T., Sakase, H., Teshigawara, K., Inoue, S., Iguchi, H., Hiramatsu, R., Leventhal, D., Takeda, K., Akira, S., and Takeuchi, M. (2004) Nat. Med. 10, 168–174
24. Haga, S., Terui, K., Zhang, H. Q., Enosawa, S., Ogawa, W., Inoue, H., Okuyama, T., Takeda, K., Akira, S., Ogino, T., Inari, K., and Ozaki, M. (2003) J. Clin. Invest. 112, 989–998
25. Sirion, J. J., and Ouchi, T. (2004) J. Biol. Chem. 279, 4066–4074
27. Sano, S., Chang, K. S., Carbajal, S., Clifford, J., Peavey, M., Kiguchi, K., Itami, S., Nickoloff, B. J., and DiGiovanni, J. (2005) Nat. Med. 11, 43–49
28. Vinkemeier, U., Cohen, S. L., Moarefi, I., Chait, B. T., Kuriyan, J., and Darnell, J. E., Jr. (1996) EMBO J. 15, 5616–5626
29. Yang, E., Henriksen, M. A., Schaefer, O., Zakharova, N., and Darnell, J. E., Jr. (2002) J. Biol. Chem. 277, 13455–13462
30. Liu, B., Mink, S., Wong, K. A., Stein, N., Getman, C., Dempsey, P. W., Wu, H., and Shuai, K. (2004) Nat. Immunol. 5, 891–898
31. Meyer, T., Marg, A., Lemke, P., Wiesner, B., and Vinkemeier, U. (2003) Genes Dev. 17, 1992–2005
32. Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Muller, M., and Decker, T. (2003) Immunity 19, 793–802
33. Kitamura, T., Onishi, M., Kinoshita, S., Shibuya, A., Miyajima, A., and Nolan, G. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9146–9150
34. Walter, M. J., Look, D. C., Tidwell, R. M., Roswit, W. T., and Holtzman, M. J. (1997) J. Biol. Chem. 272, 28582–28589
35. Sakamoto, S., Qin, J., Navarro, A., Gamero, A., Potla, R., Yi, T., Zhu, W., Baker, D. P., Feldman, G., and Larner, A. C. (2004) J. Biol. Chem. 279, 3245–3253
36. Horvath, C. M., Wen, Z., and Darnell, J. E., Jr. (1995) Genes Dev. 9, 984–994
37. Li, X., Leung, S., Qureshi, S., Darnell, J. E., Jr., and Stark, G. R. (1996) J. Biol. Chem. 271, 5790–5794
38. Lo, M. S., Brazas, R. M., and Holtzman, M. J. (2005) J. Virol. 79, 9315–9319
39. Jayaraman, S., Castro, M., O’Sullivan, M., Bragdon, M. J., and Holtzman, M. J. (1999) J. Immunol. 162, 1717–1722
40. Ota, N., Brett, T. J., Murphy, T. L., Fremont, D. H., and Murphy, K. M. (2004) Nat. Immunol. 5, 208–215
41. Zhong, M., Henriksen, M. A., Takeuchi, K., Schaefer, O., Liu, B., ten Hoeve, J., Ren, Z., Mao, X., Chen, X., Shuai, K., and Darnell, J. E., Jr. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 3966–3971
42. Bromberg, J. F., Horvath, C. M., Wen, Z., Schreiber, R. D., and Darnell, J. E., Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7673–7678
43. Mowen, K., and David, M. (2000) Mol. Cell. Biol. 20, 7273–7281
44. Kurokawa, R., Kalafus, D., Ogliastro, M.-H., Kioussi, C., Xu, L., Torchia, J., Rosenfeld, M. G., and Glass, C. K. (1998) Science 279, 700–703
45. Paulson, M., Pisharody, S., Pan, L., Guadagno, S., Mai, A. L., and Levy, D. E. (1999) J. Biol. Chem. 274, 25343–25349
46. Ting, L.-M., Kim, A. C., Cattamanchi, A., and Ernst, J. D. (1999) J. Immunol. 163, 3898–3906
47. Yuan, Z., Guan, Y., Chatterjee, D., and Chin, Y. E. (2005) Science 307, 269–273
48. Pilz, A., Ramsauer, K., Heidari, H., Leitges, M., Korvarik, P., and Decker, T. (2003) EMBO Rep. 4, 368–372
49. Yasukawa, H., Yajima, T., Duplain, H., Iwatate, M., Kido, M., Hoshijima, M., Wietzmann, M. D., Nakamura, T., Woodard, S., Xiong, D., Yoshimura, A., Chien, K. R., and Knowlton, K. U. (2003) J. Clin. Invest. 111, 469–478
50. Megidish, T., Xu, J. H., and Xu, C. W. (2002) J. Biol. Chem. 277, 8255–8259