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SHP1 Protein-tyrosine Phosphatase Inhibits gp91^{PHOX} and p67^{PHOX} Expression by Inhibiting Interaction of PU.1, IRF1, Interferon Consensus Sequence-binding Protein, and CREB-binding Protein with Homologous Cis Elements in the *CYBB* and *NCF2* Genes*

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The *CYBB* and *NCF2* genes encode the phagocyte respiratory burst oxidase proteins, gp91^{PHOX} and p67^{PHOX}. Previously, we identified homologous *CYBB* and *NCF2* cis elements that are necessary for lineage-specific transcription during late myeloid differentiation. We determined that these homologous cis elements are activated by PU.1, IRF1, interferon consensus sequence-binding protein (ICSBP), and the CREB-binding protein (CBP). Since expression of PU.1 and ICSBP is lineage-restricted, our investigations identified a mechanism of lineage-specific *CYBB* and *NCF2* transcription. Since PU.1, IRF1, ICSBP, and CBP are expressed in undifferentiated myeloid cells, our investigations did not determine the mechanism of differentiation stage-specific *CYBB* and *NCF2* transcription. In the current investigations, we determine that SHP1 protein-tyrosine phosphatase (SHP1-PTP) inhibits gp91^{PHOX} and p67^{PHOX} expression, in undifferentiated myeloid cell lines, by decreasing interaction of PU.1, IRF1, ICSBP, and CBP with the *CYBB* and *NCF2* genes. We also determine that IRF1 and ICSBP are tyrosine-phosphorylated during interferon γ differentiation of myeloid cell lines, and we identify IRF1 and ICSBP tyrosine residues that are necessary for *CYBB* and *NCF2* transcription. Therefore, these investigations identify a novel mechanism by which SHP1-PTP antagonizes myeloid differentiation and determine that tyrosine phosphorylation of IRF1 and ICSPB mediates stage-specific transcriptional activation in differentiating myeloid cells.

Myeloid differentiation is characterized by sequential acquisition of phagocyte functions, a process requiring sequential activation of myeloid-specific genes (1). We have been studying transcription of the *CYBB* and *NCF2* genes (2–7). These genes, which encode the respiratory burst oxidase proteins gp91^{PHOX} and p67^{PHOX}, are actively transcribed in myeloid cells that have differentiated beyond the promyelocyte stage (8, 9). In mature phagocytes, *CYBB* and *NCF2* transcription is increased by IFNγ,[1] lipopolysaccharide, and tumor necrosis factor α during the inflammatory response (10). Therefore, *CYBB* and *NCF2* transcription is lineage-specific and differentiation stage-specific and increases during phagocyte activation. Since *CYBB* and *NCF2* transcription occurs simultaneously, we hypothesize that common transcription factors regulate expression of these two genes (6, 7).

Previous investigations (7, 11) have identified positive and negative cis elements that regulate *CYBB* and *NCF2* transcription. In undifferentiated myeloid cells, *CYBB* transcription is repressed by interaction of HoxA10 and Pbx1a with three negative cis elements in the *CYBB* promoter. Consistent with our hypothesis, several homologous sequences in the *NCF2* gene also interact *in vitro* with HoxA10 and Pbx1a (7). We found that HoxA10 is tyrosine-phosphorylated during IFNγ-induced differentiation of myeloid cell lines, and tyrosine phosphorylation decreases HoxA10 DNA binding affinity (7). Therefore, post-translational modification of HoxA10 is one mechanism that regulates differentiation stage-specific gp91^{PHOX} and p67^{PHOX} expression (7).

We also identified homologous, positive cis elements in the *CYBB* and *NCF2* genes that are necessary for lineage-specific, IFNγ-induced transcription (5, 6). These cis elements (referred to as "HAF1" elements for "hematopoiesis-associated factors") are proximate to transcription start sites in the two genes. We found that the HAF1 elements are activated by PU.1 (an ETS protein) and two interferon regulatory factors (IRFs), interferon regulatory factor 1 (IRF1) and the interferon consensus sequence-binding protein (ICSBP) (5, 6). These proteins cooperate to recruit the CREB-binding protein (CBP) to the proximal *CYBB* and *NCF2* promoters (6). *In vitro*, PU.1 binds the HAF1 elements as a monomer, as a heterodimer with either IRF1 or ICSBP (the "HAF1" complex), or as a multiprotein complex with IRF1 and ICSBP and CBP (the "HAF1a" complex) (6). Although the HAF1 cis elements contain composite ETS/IRF consensus sequences, IRF1 and ICSBP do not bind these elements in the absence of PU.1 (6). This suggests that the IRF proteins interact with both the HAF1-DNA sequence and PU.1 protein domains. Additionally, PU.1, IRF1, and ICSBP do not interact with CBP in the absence of a HAF1 DNA-binding site (6). This suggests that CBP interacts with PU.1, IRF1, and/or ICSBP domains that are brought into proximity by DNA binding.

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[1] The abbreviations used are: IFNγ, interferon γ; SHP1-PTP, SHP1 protein-tyrosine phosphatase; PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase; bp, base pair; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; ICSBP, interferon consensus sequence-binding protein; IRFs, interferon regulatory factors.

Since expression of PU.1 and ICSBP is restricted to myeloid and B-cells, our previous investigations (5, 6) identified a mechanism that contributes to lineage-restricted *CYBB* and *NCF2* transcription. However, these investigations did not determine the mechanism by which PU.1, IRF1, and ICSBP participate in differentiation stage-specific transcription. By *in vitro* DNA binding assays, we determined that access to the HAF1 elements is not sterically hindered by binding of HoxA10/Pbx1a to the *CYBB* and *NCF2* repressor elements, in undifferentiated myeloid cells (2, 3). Additionally, we determined that IFNγ treatment of myeloid cell lines does not increase the abundance of PU.1, IRF1, ICSBP, or CBP. This is consistent with previous results (1, 12, 13) indicating that PU.1 and ICSBP regulate transcription at multiple points during myelopoiesis. Based on these observations, we hypothesize that post-translational modification of proteins that participate in the HAF1a DNA-protein complex occurs during myelopoiesis and alters the HAF1a protein-DNA interaction.

Previously, other investigators (14–16) demonstrated that SHP1 protein-tyrosine phosphatase (SHP1-PTP) antagonizes phagocyte differentiation and activation. In the current investigations, we demonstrate that SHP1-PTP activity decreases *CYBB* and *NCF2* transcription, in undifferentiated myeloid cell lines. Consistent with this, SHP1-PTP decreases DNA binding of the HAF1a protein complex and tyrosine phosphorylation of IRF1 and ICSBP. We also determine that activation of the HAF1 cis elements is dependent upon tyrosine phosphorylation of conserved tyrosine residues in IRF1 and ICSBP. Therefore, these investigations identify modulation of transcription factor interaction with the *CYBB* and *NCF2* genes as a mechanism by which SHP1-PTP regulates phagocyte differentiation and activation. These investigations also demonstrate that tyrosine phosphorylation of IRF1 and ICBP is necessary for these proteins to activate oxidase gene transcription during myeloid differentiation.

MATERIALS AND METHODS

Plasmids and Site-directed Mutagenesis

Genomic Clones and Reporter Constructs—CYBB promoter sequences have been described previously (17). *NCF2* genomic clone was obtained from T. Leto (National Institutes of Health, Bethesda) (18). *NCF2* reporter gene constructs were described previously (6). *CYBB* and *NCF2* genomic sequences were subcloned into the reporter gene vector pCATE (Promega, Madison, WI) as described (6). Artificial promoter/reporter constructs with multiple copies of the *CYBB* or *NCF2* HAF1 cis elements have been described previously (5, 6) using the minimal promoter/reporter vector, p-TATACAT (19) (obtained from Dr. A. Kraft, University of Colorado, Denver, CO).

Plasmids for Protein Expression—The cDNA for human PU.1 was obtained from M. Klemsz (Indiana University, Indianapolis) and subcloned in to the mammalian expression vector pSRα (20, 21). The human ICSBP cDNA, obtained from B.-Z. Levi (Technicon, Haifa, Israel), and the human IRF1 cDNA, obtained from R. Pine (New York University Medical Center, New York, NY), were subcloned into the mammalian expression vectors pcDNAamp and pcDNA3.1his (Invitrogen, San Diego, CA). The pcDNA3.1his vector expresses fusion proteins with the "xpress" and 6× histidine epitope tags. The cDNAs for mutant IRF1 (mutation of tyrosine 109 to phenylalanine) and ICSBP (mutation of tyrosine 95 to phenylalanine) were generated by site-directed mutagenesis, using the CLONTECH "Quickchange" protocol. Mutant cDNAs were sequenced to verify mutagenesis and to determine that no other mutations had been introduced. Y109F IRF1 and Y95F ICSBP were also subcloned into the pcDNAamp and pcDNA3.1his vectors. The cDNAs for SHP1-PTP and CS453-SHP1-PTP (dominant negative), subcloned into the pSRα vector, were obtained from Dr. Stuart Frank (Birmingham Veterans Affairs Hospital and the Department of Medicine, University of Alabama, Birmingham) (23).

Oligonucleotides

Oligonucleotides were synthesized by the Core Facility of the Comprehensive Cancer Center, University of Alabama, Birmingham, or by the Oligonucleotide Core Facility at the Riley Children's Hospital of Indiana, Indianapolis. The oligonucleotides used are as follows: *CYBB* promoter HAF1 cis element from −32 to −69 bp (cybbhaf) (2), 5′-ctgctgttttcatttcctcattggaagaagaagcatag-3′; *NCF2* intron 1 HAF1 cis element from 160 to 190 bp 5′ of the ATG (ncf2haf) (6), 5′-ccaaaaggtgggacatttcctgtggatttgc-3′; CCAAT box from the α-globin gene (urccaat) (24), 5′-ccgggctccgcgccagccaatgagcgccgcgg-3′.

Cell Culture

All cell lines were of human origin. The myelomonocytic cell line U937 (24) was obtained from Andrew Kraft (University of Colorado, Denver, CO). The promyelocytic cell line PLB985 (25) was obtained from Tom Rado (University of Alabama, Birmingham). Cell lines were maintained and differentiated as described (6). U937 cells were treated with 200 or 1,000 units per ml human recombinant IFNγ (Roche Molecular Biochemicals).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extract proteins were prepared by the method of Dignam *et al.* (26) with protease and inhibitors, as described previously (6). In some experiments, protein-tyrosine phosphatase inhibitor was added to the buffers, as described (0.1 μM $NaVO_3$) (5, 6). Oligonucleotides probes were prepared, and EMSA and antibody supershift assays were performed, as described (5). EMSA were performed as previously described previously (5). In some experiments, the nuclear proteins were preincubated with *Yop* protein-tyrosine phosphatase or purified SHP1 protein for 30 min at 30 °C, as described by the manufacturer's instructions (New England Biolabs, Worchester, MA). Control samples were similarly incubated in the absence of protein-tyrosine phosphatase. In other experiments, EMSA binding reactions were preincubated for 4 h on ice with anti-xpress and anti-His_6 antibodies prior to addition of radiolabeled probe (0.5 μg each, Santa Cruz Biotechnology, Santa Cruz, CA).

Stable U937 Transfectants

To generate stable transfectant pools, U937 cells (32×10^6) were transfected with 50 μg of plasmid: SHP1/pSRα, CS453-SHP1/pSRα, or control pSRα; IRF1/pcDNA3.1his, Y109F IRF1/pcDNA3.1his, ICSBP/pcDNA3.1his, Y95F ICSBP/pcDNA3.1his, or control pcDNA3.1his. Cells were incubated in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1% penicillin/streptomycin, 1 mg/ml geneticin (G418) to select for stable transfectants. Expression of the various proteins was verified by Western blots of cell lysates, according to standard procedures, with commercially available antibodies.

Transfection and Reporter Gene Assays

Cells were transfected by electroporation, as described (5). Stable U937 transfectants (with SHP1/pSRα, CS453-SHP1/pSRα, or pSRα) were co-transfected with 50 μg of pCATE constructs and 15 μg p-CMV/β-galactosidase (CLONTECH, Palo Alto, CA). In other experiments, U937 cells were transfected with 70 μg of p-TATACAT or p-cybbhafTATACAT; 30 μg of pSRα or PU.1/pSRα; 30 μg of IRF1/pcDNAamp, Y109F IRF1/pcDNAamp, ICSBP/pcDNAamp, Y95F ICSBP/pcDNAamp, or control pcDNAamp; or 15 μg each of various combinations of IRF1/pcDNAamp or Y109F IRF1/pcDNAamp with ICSBP/pcDNAamp or Y95F ICSBP/pcDNAamp; and 15 μg of p-CMV/β-galactosidase. Transfectants were incubated for 24 h at 37 °C, 5% CO_2, followed by 24 h with or without IFNγ (1,000 units/ml). Preparation of cell extracts, β-galactosidase, and chloramphenicol acetyltransferase assays (CAT) assays were as described (27, 28).

Northern Blots and RNA Stability Assays

Total cellular RNA was extracted (29) from U937 cells with or without 48 h of IFNγ treatment (200 units/ml). Northern blots were performed with 10 or 20 μg of RNA, as described (30). For analysis of RNA stability, U937 cells were treated with actinomycin D (10 μg/ml). Cells were harvested at various times, and RNA was extracted for Northern blot. Autoradiographs of Northern blots were analyzed by densitometry to determine mRNA abundance at various times after actinomycin D treatment. Results were normalized to mRNA abundance at $t = 0$.

SHP1 Immunoprecipitation and Protein-tyrosine Phosphatase Assays

U937 stable transfectants with SHP1, CS453-SHP1, or control vector were lysed in RIPA buffer, as described (in the presence of SDS) (6). Cell lysates (200 μg) were immunoprecipitated for 4 h at 4 °C with anti-SHP1 antibody (2.0 μg, Santa Cruz Biotechnology, Santa Cruz, CA) or irrelevant control antibody (anti-mouse IgG), and immunoprecipitates

were collected with staphylococcus protein A-Sepharose, as described previously (6). For PTP assays, staphylococcus protein A-Sepharose beads were washed extensively in RIPA buffer (with no SDS) and resuspended in Tyr(P) assay buffer (25 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA). Phosphopeptide RRLIEDAEpYAARG (where pY is Tyr(P)) (2 nM) was added, and the samples were incubated for 15 min at room temperature. Assays were performed according to Harder *et al.* (31) using a commercially available kit (PTP Assay Kit 1, Upstate Biotechnology, Inc., Lake Placid, NY). In other experiments, immunoprecipitated SHP1 (or control immunoprecipitate) was eluted with pH 4.0 buffer, neutralized with 1 M Tris, pH 9.5 (as per the manufacturer, Amersham Pharmacia Biotech), and incubated with nuclear proteins or *in vitro* translated proteins.

Immunoprecipitation and Western Blotting

Immunoprecipitation experiments were performed with 200 μg of nuclear proteins extracted from U937 cells, with or without 48 h IFNγ incubation. Nuclear proteins were diluted into RIPA buffer, with protease and phosphatase inhibitors, as described previously (6), and incubated with either 1 μl of anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Inc., Lake Placid, NY), anti-ICSBP antibody (goat anti-human ICSBP, Santa Cruz Biotechnology), anti-IRF1 antibody (mouse anti-human IRF1, Santa Cruz Biotechnology), or irrelevant antibody (mouse anti-rabbit IgG or goat anti-rabbit IgG, Santa Cruz Biotechnology) for 4 h at 4 °C, followed by 1 h of incubation with 15 μl of 50% staphylococcus protein A-Sepharose bead slurry. In some experiments, immunoprecipitations were performed in the presence of SDS to prevent co-immunoprecipitation of other proteins. Beads were washed with RIPA buffer (no SDS), proteins eluted in SDS sample buffer, separated on 12% SDS-PAGE, and transferred to nitrocellulose. Blots were probed with antibodies as indicated under "Results," and proteins were detected by chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). Immunoprecipitation of *in vitro* translated proteins was similarly performed.

In Vitro Translated Proteins

In vitro transcription and translation of ICSBP, IRF-1, Y95F ICSBP, and Y109F IRF1 (sub-cloned into pcDNAamp) were performed using the Promega *In Vitro* Transcription System and rabbit reticulocyte lysate *In Vitro* Translation System, according to the manufacturer's instructions (Promega, Madison, WI).

GST Fusion Protein "Pull-down" Assays

JM109 *Escherichia coli* cells transformed with PU.1/pGEX2, IRF1/pGEX1, or control pGEX2 were grown to log phase, supplemented to 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated for 3 h at 37 °C with shaking. The cells were harvested and resuspended in HN buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 5 mM NaF), and sonicated on ice. Debris was removed by centrifugation, and the lysate was incubated 30 min at 4 °C with glutathione-agarose beads (Sigma) and washed extensively with HN buffer. The beads were preincubated for 30 min at 4 °C with 5 μl of control rabbit reticulocyte lysate and then for 1 h with 20 μl of [^{35}S]methionine-labeled and *in vitro* translated protein and washed extensively in HN buffer. Proteins were eluted with SDS-PAGE sample buffer and separated on 12% SDS-PAGE, and an autoradiograph was performed.

RESULTS

Protein-tyrosine Phosphatase Activity Inhibits Interaction of PU.1, IRF1, ICSBP, and CBP with the CYBB and NCF2 Genes—Previously, we determined that lineage and differentiation stage-specific *CYBB* and *NCF2* transcription requires intact HAF1 cis elements in the two genes (2, 6). We also determined that overexpression of PU.1, IRF1, ICSBP, and CBP activates artificial promoter constructs with multiple copies of the *CYBB* or *NCF2* HAF1 elements in myeloid cell line transfectants (6). Additionally, we found that this interaction is increased by IFNγ-induced differentiation of the transfectants. Consistent with the functional data, PU.1, IRF1, ICSBP, and CBP bind to the *CYBB* and *NCF2* cis elements in EMSA (5, 6). Three protein complexes bind *in vitro* to the *CYBB* and *NCF2* HAF1 cis elements as follows: PU.1 monomer; PU.1 heterodimer with IRF1 or ICSBP (the "HAF1" complex); and a multiprotein complex that includes PU.1, IRF1, ICSBP, and CBP (the "HAF1a" complex) (see Fig. 1) (5, 6). Since IRF1 and ICSBP binding to the HAF1 cis elements requires PU.1 (6), and since ICSBP and IRF1 are able to interact with each other (32), three of the following interactions are possible: PU.1 interacts directly with IRF1 and IRF1 binds ICSBP; PU.1 interacts directly with ICSBP and ICSBP binds IRF1; or PU.1 interacts simultaneously with IRF1 and ICSBP. We found that DNA binding of the three proteins creates a binding site for CBP (6).

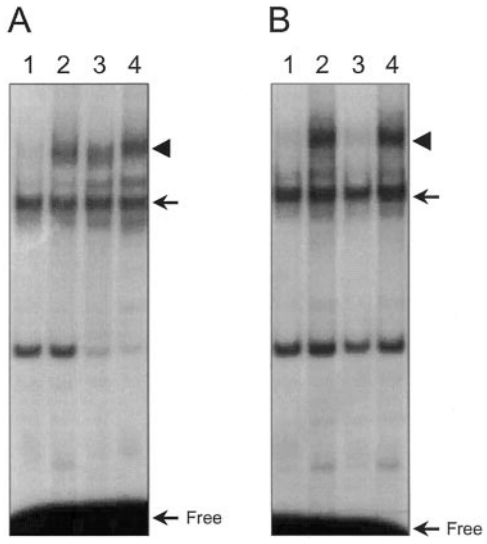

FIG. 1. **PTP activity antagonizes *in vitro* binding of the HAF1a complex to the *CYBB* HAF1 cis element.** *A,* treatment of U937 cells with sodium orthovanadate increases binding of the HAF1a complex to the *CYBB* HAF1 cis element. Nuclear proteins were isolated from U937 cells with or without sodium orthovanadate treatment (a PTP inhibitor) and with or without IFNγ treatment. EMSA was performed with the *CYBB* HAF1 cis element probe, and nuclear proteins were isolated under the following conditions: *lane 1,* no sodium orthovanadate, no IFNγ; *lane 2,* no sodium orthovanadate, 48 h of IFNγ; *lane 3,* sodium orthovanadate, no IFNγ; *lane 4,* sodium orthovanadate, 48 h of IFNγ. The HAF1 complex is indicated with an *arrow* and the HAF1a complex with an *arrowhead.* The fast migrating complex is PU.1 monomer. PTP inhibitor treatment of U937 cells induces almost as much HAF1a binding as IFNγ differentiation. In contrast, HAF1 complex and PU.1 monomer binding are not altered. *B,* treatment of nuclear proteins from differentiated U937 cells with *Yop* tyrosine phosphatase decreases HAF1a complex binding. Nuclear proteins were isolated from U937 cells, with or without IFNγ treatment. U937 nuclear proteins were incubated with or without *Yop* protein-tyrosine phosphatase. EMSA were performed with the *CYBB* HAF1 cis element probe and nuclear proteins under the following conditions: *lane 1,* no IFNγ, no *Yop*; *lane 2,* 48 h ofIFNγ, no *Yop*; *lane 3,* 48 h of IFNγ, *Yop*; *lane 4,* 48 h of IFNγ, sham incubation in *Yop* buffer. The HAF1 complex is indicated by the *arrow,* and the HAF1a complex by the *arrowhead. Yop* treatment decreases binding of the HAF1a complex but does not affect the HAF1 complex or PU.1 monomer.

In our previous investigations (5, 6), IFNγ differentiation of myeloid cell lines did not increase *in vitro* binding of either PU.1, the HAF1 complex, or the HAF1a complex to the *CYBB* and *NCF2* cis elements. Therefore, our transfection data indicate that the HAF1 cis elements are necessary for *CYBB* and *NCF2* transcription during IFNγ-induced differentiation in myeloid cell lines. In contrast, our binding assays indicate that *in vitro* protein interaction with the HAF1 elements is not altered by IFNγ-induced differentiation of myeloid cell lines. To resolve this discrepancy, we re-evaluated the conditions under which we were analyzing *in vitro* DNA-protein interactions.

We first noted the HAF1a complex in EMSA with the *CYBB* HAF1 cis element and myeloid nuclear proteins that were isolated in the presence of protein phosphatase inhibitors (sodium orthovanadate and sodium fluoride) (5). In these previous experiments, phosphatase inhibitors were added to all buffers

used for nuclear protein isolation, including a 10-min incubation in pre-lysis buffer (according to Dignam *et al.* (26)). Therefore, our previous investigations assumed that protein phosphatase activity is equivalent in IFNγ-differentiated and -undifferentiated myeloid cell lines. If IFNγ treatment decreases net protein phosphatase activity, this assumption would be incorrect. In that case, treatment of the cells with protein phosphatase inhibitors might obscure IFNγ-induced alterations in protein phosphorylation and therefore IFNγ-induced alteration in protein-protein or protein-DNA interactions. To investigate this, we determined whether protein-tyrosine phosphatase (PTP) activity influences *in vitro* protein binding to the HAF1 cis elements.

U937 cells were incubated for 48 h, with or without IFNγ, and nuclear proteins were extracted, with or without pretreatment with the protein-tyrosine phosphatase inhibitor sodium orthovanadate (0.1 μM for 15 min). EMSA were performed with a HAF1-binding probe, as described previously (5). In the absence of PTP inhibitor, HAF1a binding is minimal in EMSA with nuclear proteins from undifferentiated U937 cells and increases in binding assays with nuclear proteins from IFNγ-differentiated cells (Fig. 1*A*). However, we found that sodium orthovanadate treatment of undifferentiated U937 cells also increases *in vitro* HAF1a binding. Additionally, IFNγ differentiation does not significantly increase HAF1a complex binding in nuclear proteins from PTP inhibitor-treated U937 cells (Fig. 1*A*). Therefore, HAF1a binding is approximately equivalent in EMSA with nuclear proteins from IFNγ-differentiated U937 cells, PTP inhibitor-treated undifferentiated U937 cells, and PTP-inhibitor-treated, IFNγ-differentiated U937 cells. These results suggest that PTP activity inhibits interaction of the HAF1a proteins in undifferentiated U937 cells. In contrast, IFNγ and sodium orthovanadate do not alter binding of PU.1 monomer or the HAF1 complex to the HAF1 cis elements *in vitro*.

To investigate further the influence of PTP activity on *in vitro* protein binding to the *CYBB* and *NCF2* HAF1 cis elements, we incubated nuclear proteins from IFNγ-differentiated U937 cells with *Yop* protein-tyrosine phosphatase. Control nuclear proteins were sham-incubated in the same buffer, in the absence of *Yop*. The nuclear proteins used in this experiment were isolated from U937 cells that were not sodium orthovanadate-treated. We found that *Yop* treatment of nuclear proteins decreases binding of the HAF1a complex to HAF1 DNA probes but does not alter binding of the HAF1 complex, or PU.1 (Fig. 1*B*). These results suggest that the HAF1a protein-protein-DNA interaction is dependent on protein-tyrosine phosphorylation.

SHP1-PTP Activity Decreases CYBB and NCF2 Transcription by the HAF1a Proteins—We hypothesize that protein-tyrosine phosphatase activity, in undifferentiated myeloid cell lines, inhibits protein interaction with the *CYBB* and *NCF2* HAF1 cis elements, thereby decreasing gp91^{PHOX} and p67^{PHOX} expression. We considered SHP1 a candidate PTP, based upon the phenotype of SHP1 −/− (viable moth-eaten) mice (14, 15). These mice have an increase in mature, circulating phagocytes and die from progressive pulmonary inflammation. Consistent with this, undifferentiated U937 cells that are overexpressing dominant negative SHP1 (CS453-SHP1) manifest respiratory burst activity (16). Since gp91^{PHOX} and p67^{PHOX} are expressed at low levels in undifferentiated U937 cells, we hypothesized that SHP1-PTP inhibition increases *CYBB* and *NCF2* transcription in undifferentiated U937 cells (33). We also hypothesized that IFNγ-induced decrease in SHP1-PTP activity might contribute to signal-dependent increase in *CYBB* and *NCF2* in transcription, in differentiating U937 cells.

Therefore, we investigated SHP1-PTP activity during IFNγ-

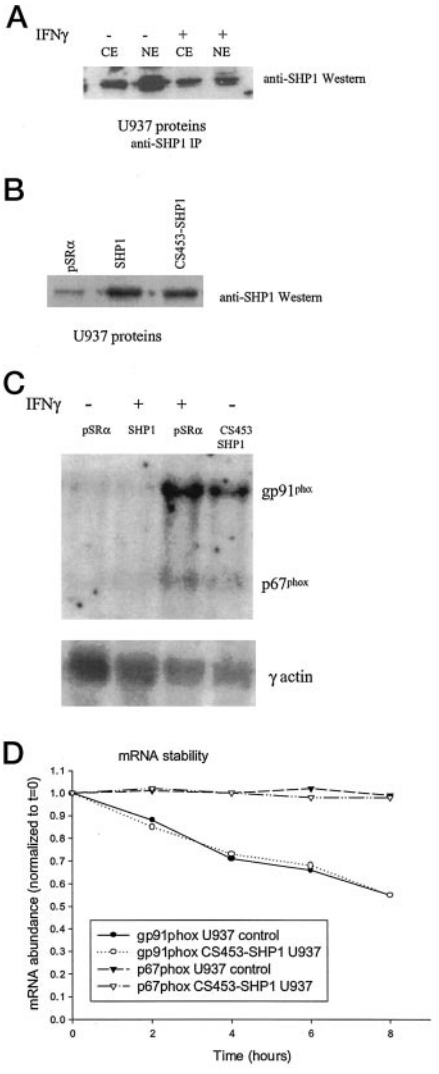

FIG. 2. **Inhibition of SHP1-PTP activity increases gp91^{PHOX} and p67^{PHOX} mRNA abundance.** *A,* SHP1-PTP protein is predominantly nuclear in U937 cells, and protein abundance decreases during IFNγ-induced differentiation. U937 cells were incubated for 48 h with or without IFNγ differentiation. Nuclear and cytoplasmic proteins were isolated and separated by SDS-PAGE (50 μg), and Western blots were probed with anti-SHP1 antibody. Nuclear (*NE*) and cytoplasmic (*CE*) fractions and IFNγ treatment are as indicated. IFNγ treatment results in modest decrease in nuclear SHP1 protein. *B,* SHP1 and CS453-SHP1 are overexpressed in U937 stable transfectants. U937 cells were stably transfected with vectors to overexpress SHP1, dominant negative SHP1 (CS453), or control pSRα vector. Cell lysates were separated by SDS-PAGE (10 μg) and probed with anti-SHP1 antibody. Increased immunoreactive SHP1 is present in cells overexpressing either SHP1 or CS453-SHP1 as indicated. *C,* inhibition of SHP1-PTP in U937 cells increases gp91^{PHOX} and p67^{PHOX} mRNA abundance. U937 cells were stably transfected with SHP1, a dominant negative SHP1 (CS453), or control vector (pSRα). RNA was isolated, with or without 48-h IFNγ-induced differentiation, and analyzed by Northern blot for gp91^{PHOX} and p67^{PHOX} expression, as indicated. Blots were also probed for γ-actin, to control for RNA loading. SHP1 overexpression decreases gp91^{PHOX} and p67^{PHOX} mRNA abundance in differentiated U937 cells. Consistent with this, SHP1 inhibition increases gp91^{PHOX} and p67^{PHOX} mRNA in undifferentiated U937 cells. *D,* SHP1-PTP inhibition does not increase gp91^{PHOX} and p67^{PHOX} mRNA stability in U937 cells. U937 stable transfectants with CS453-SHP1 or vector control were treated with actinomycin D (10 μg/ml), and total RNA was isolated at t = 0, 2, 4, 6, and 8 h. RNA was analyzed by Northern blots probed for gp91^{PHOX}, p67^{PHOX}, and γ-actin (control message). MRNA abundance was determined by densitometry of Northern blot autoradiographs. Results are expressed relative to gp91^{PHOX} and p67^{PHOX} mRNA abundance at t = 0 and are normalized for γ-actin mRNA to control for loading. Data are shown for a representative blot. Although CS453-SHP1 overexpression increases abundance of gp91^{PHOX} and p67^{PHOX} mRNA, there is no change in the stability of the messages.

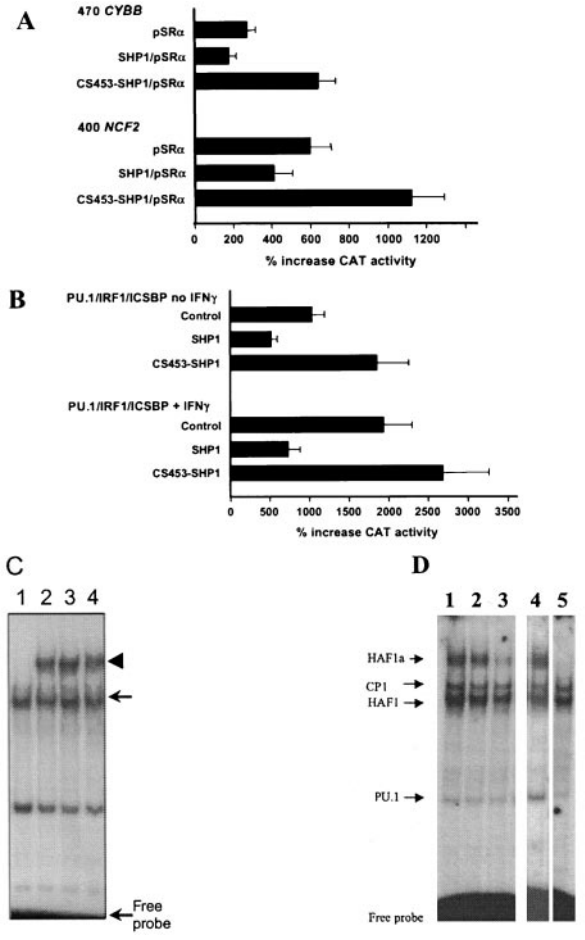

FIG. 3. **Inhibition of SHP1-PTP activity increases *CYBB* and *NCF2* transcription via the homologous HAF1 cis elements.** *A,* SHP1-PTP inhibition increases expression from reporter constructs with 470 bp of the *CYBB* promoter or 400 bp of the *NCF2* promoter. U937 stable transfectants overexpressing SHP1, CS453-SHP1, or empty vector (pSRα) control were co-transfected with reporter constructs with either the proximal 470 bp of *CYBB* promoter, the proximal 400 bp of the *NCF2* 5′ flank, or empty vector control (p-CATE). Reporter gene assays were performed 48 h after transfection. Results are expressed as percent increase in CAT activity relative to empty pCATE vector. Overexpression of CS453-SHP1 significantly increases reporter expression from the *CYBB* promoter construct (143 ± 12.8%, $n = 3$, $p = 0.026$) and the *NCF2* promoter construct (129 ± 10.4%, $n = 3$, $p = 0.056$). Consistent with this, SHP1 overexpression decreases expression from the *CYBB* promoter (41.8 ± 6.6%, $n = 3$, $p = 0.174$) and *NCF2* promoter (39.4 ± 5.5%, $n = 3$, $p = 0.196$). Neither protein alters pCATE control vector expression. *B,* SHP1-PTP inhibition increases activation of the *CYBB* HAF1 cis element by PU.1, IRF1, and ICSBP. U937 stable transfectants overexpressing SHP1, CS453-SHP1, or empty vector (pSRα) control were co-transfected with vectors to overexpress PU.1, IRF1, and ICSBP and either an artificial promoter construct with four copies of the *CYBB* HAF1 cis element (p-cybbhafTATACAT) or empty vector control (p-TATACAT). Transfectants were incubated 48 h, with or without IFNγ, and reporter assays were performed. Results are expressed as percent increase in CAT activity relative to p-TATACAT vector control. Overexpression of CS453-SHP1 increases activation of the HAF1 cis element by PU.1, IRF1, and ICSBP in undifferentiated (79.6 ± 17.7%, $n = 5$, $p = 0.027$) and in IFNγ-treated U937 transfectants (38.6 ± 13.4%, $n = 3$, $p = 0.103$). Consistent with this, overexpression of SHP1 significantly decreases activation of the *CYBB* HAF1 cis element by PU.1, IRF1, and ICSBP in undifferentiated (49.3% + 13.3%, $n = 5$, $p = 0.026$) and in IFNγ-treated U937 transfectants (61.8 ± 19.6%, $n = 3$, $p = 0.037$). In contrast, overexpression of these proteins does not alter p-TATACAT expression. *C,* overexpression of CS453-SHP1 in U937 cells increases *in vitro* binding of the HAF1a complex to the *CYBB* HAF1 cis element. Nuclear proteins were isolated from U937 cells that were stably transfected with CS453-SHP1 or empty vector control (pSRα), with and without IFNγ-differentiation. EMSA was performed with the *CYBB* HAF1 cis element probe and nuclear proteins as indicated: *lane 1,* pSRα control, no IFNγ; *lane 2,* CS453-SHP1/pSRα, no IFNγ; *lane 3,* pSRα control, 48 h of IFNγ; *lane 4,* CS453-SHP1/pSRα, 48 h of IFNγ. The HAF1 complex is indicated by the *arrow* and the HAF1a complex by the *arrowhead.* SHP1-PTP inhibition increases binding of the HAF1a complex, but not the HAF1 complex, to the *CYBB* cis element. *D,* SHP1-PTP activity decreases HAF1a complex binding to the *CYBB* HAF1 cis elements. EMSA was performed with the *CYBB* HAF1 cis element probe and either nuclear proteins isolated from IFNγ-treated U937 cells that were incubated as follows: *lane 1,* nothing; *lane 2,* control eluate; *lane 3,* purified SHP1 or nuclear proteins from IFNγ-treated U937 cells transfected with control pSRα (*lane 4*), SHP1/pSRα (*lane 5*). The HAF1a, HAF1, and PU.1 complexes are indicated. Incubation with immunoprecipitated SHP1 and overexpression of SHP1 both decrease binding of the HAF1a complex in nuclear proteins from IFNγ-treated U937 cells.

induced differentiation of U937 cells. SHP1 was immunoprecipitated from U937 lysates and used in quantitative PTP assays. We found that IFNγ treatment of U937 cells decreases SHP1-PTP activity by 46.1 ± 6.7% ($n = 3$, $p = 0.017$). This result was of interest because total PTP activity significantly increases in IFNγ-treated U937 cells (326 ± 30%, $n = 6$, $p < 0.01$). Consistent with the results of our PTP assays, IFNγ treatment of U937 cells modestly decreases in SHP1 protein abundance (Fig. 2*A*). Therefore, this temporal relationship is consistent with our hypotheses.

To investigate the role of SHP1-PTP in *CYBB* and *NCF2* transcription, U937 cells were stably transfected with a vector to overexpress SHP1, CS453-SHP1, or vector control. Overexpression of SHP1 and CS453-SHP1 was verified by Western blots (Fig. 2*B*). To determine the functional effects of the overexpressed proteins, SHP1 was immunoprecipitated from the transfectants for protein-tyrosine phosphatase assays. We found that SHP1-PTP activity in SHP1-overexpressing transfectants is 34.5 ± 2.2% greater than control transfectants ($n = 6$, $p = 0.048$). In CS453-SHP1 U937 transfectants, SHP1-PTP activity is 49.0 ± 2.9% less than control transfectants ($n = 6$, $p < 0.01$), which is similar to the IFNγ-induced decrease in SHP1-PTP activity. Therefore, we used these stable U937 transfectants to investigate the effect of SHP1 on gp91^{PHOX} and p67^{PHOX} message abundance.

We performed Northern blots of RNA isolated from these U937 stable transfectants with and without IFNγ-induced differentiation. In undifferentiated U937 cells, we found that overexpression of CS453-SHP1 increases gp91^{PHOX} and p67^{PHOX} mRNA abundance relative to control vector transfectants (Fig. 2*C*). Consistent with this, overexpression of SHP1 decreases gp91^{PHOX} and p67^{PHOX} mRNA abundance in IFNγ-treated U937 transfectants. These results suggest that SHP1-PTP activity decreases either *CYBB* and *NCF2* transcription, or gp91^{PHOX} and p67^{PHOX} mRNA stability, or both. Therefore, we investigated the effect of SHP1-PTP on gp91^{PHOX} and p67^{PHOX} mRNA stability in undifferentiated U937 cells. RNA was isolated from U937 stable transfectants with CS453-SHP1 or control vector, incubated for various times in actinomycin D (to inhibit RNA synthesis). We found that CS453-SHP1 overexpression does not alter the 8-h half-life of gp91^{PHOX} mRNA in undifferentiated U937 cells (Fig. 2*D*, $p = 0.84$). Similarly, SHP1-PTP inhibition does not alter stability of the much more stable p67^{PHOX} message. Since SHP1-PTP inhibition did not increase gp91^{PHOX} or p67^{PHOX} mRNA stability in undifferentiated U937 cells, we investigated the impact on *CYBB* and *NCF2* transcription.

Previously (5, 6), we identified specific sequences in the promoter regions of the *CYBB* and *NCF2* genes that mediate IFNγ-induced transcription of these two genes. We found that reporter constructs with either 470 bp of *CYBB* promoter or 400 bp of *NCF2* 5′ flank demonstrate IFNγ-inducible transcription in U937 cells (these constructs are referred to as 470cybb/pCATE and 400ncf2/pCATE, respectively) (6). Importantly,

IFNγ differentiation of U937 cells induces equivalent increase in 470cybb/pCATE reporter expression and endogenous gp91^{PHOX} mRNA and in 400ncf2/pCATE reporter expression and endogenous p67^{PHOX} mRNA (6). Therefore, we transfected these constructs into U937 stable transfectants with CS453-SHP1, SHP1, or empty vector control. We found that overexpression of CS453-SHP1 significantly increases reporter expression from the *CYBB* and *NCF2* promoters (Fig. 3*A*). Reporter expression from the 470-bp *CYBB* construct was 143 ± 12.8% greater in CS453-SHP1 U937 transfectants than in control U937 transfectants ($n = 3$, $p = 0.026$). Similarly, overexpression of CS453-SHP1 increases reporter expression from the 400-bp *NCF2* construct by 129 ± 10.4%, relative to control U937 transfectants ($n = 3$, $p = 0.056$). Consistent with this, basal expression of these reporter constructs is decreased by SHP1 overexpression in U937 cells, 41.8 ± 6.6% decrease in reporter expression in 470cybb/pCATE transfectants ($n = 3$, $p = 0.174$) and 39.4 ± 5.5% decrease in 400ncf2/pCATE transfectants ($n = 3$, $p = 0.196$). In contrast, these proteins have no effect on empty, control pCATE vector reporter expression.

We next investigated whether SHP1-PTP influences *CYBB* and *NCF2* transcription via the HAF1 cis elements. U937 cells stable transfectants with SHP1, CS453-SHP1, or empty vector control were co-transfected with an artificial promoter construct with multiple copies of the *CYBB* HAF1-binding site (referred to as p-cybbhafTATACAT) and vectors to overexpress PU.1, IRF1, and ICSBP. These proteins synergistically activate artificial promoter/reporter constructs with multiple copies of the *CYBB* or *NCF2* HAF1 cis elements, and transcriptional activation is increased by IFNγ (5, 6). We found that overexpression of CS453-SHP1 increases reporter expression from p-cybbhafTATACAT by 79.6% in undifferentiated U937 cells (relative to vector control transfectants, $n = 5$, $p = 0.027$) (Fig. 3*B*). Overexpression of CS453-SHP1 in IFNγ-treated U937 transfectants increases reporter expression by 38.6% ($n = 3$, $p = 0.103$). In contrast, overexpression of SHP1 decreases p-cybbhafTATACAT expression by 49.3% ($n = 5$, $p = 0.026$) in undifferentiated U937 cells and by 61.8% ($n = 3$, $p = 0.037$) in IFNγ-differentiated U937 transfectants. Interestingly, CS453-SHP1 overexpression and IFNγ treatment have an equivalent effect on p-cybbTATACAT reporter expression in U937 cells co-transfected with PU.1, IRF1, and ICSBP (reporter activity not significantly different, $p = 0.48$).

SHP1-PTP Decreases HAF1a Protein Complex Interaction with the CYBB and NCF2 Promoters—Based upon the results of these transfection experiments, we investigated the impact of SHP1 inhibition on *in vitro* protein binding to the *CYBB* HAF1 cis element. We found that CS453-SHP1 overexpression increases HAF1a complex binding, in EMSA with a HAF1 cis element DNA probe and nuclear proteins from undifferentiated U937 transfectants (Fig. 3*C*). Consistent with this, HAF1a complex binding decreases in EMSA with nuclear proteins from IFNγ-treated, SHP1-overexpressing U937 cells (Fig. 3*D*). To investigate this further, we incubated nuclear proteins from IFNγ-treated U937 cells with either SHP1 (immunoprecipitated from undifferentiated U937 lysates) or negative control (irrelevant, rabbit anti-mouse IgG immunoprecipitate). We found that SHP1 treatment of nuclear proteins decreases *in vitro* binding of the HAF1a complex but not the HAF1 complex or PU.1 (Fig. 3*D*). These results suggest that SHP1-PTP activity impacts tyrosine phosphorylation of one or more of the HAF1a proteins.

IFNγ Differentiation of U937 Cells Is Associated with IRF1 and ICSBP Tyrosine Phosphorylation—These experiments suggest that tyrosine phosphorylation of IRF1, ICSBP, or CBP increases the affinity of the protein-protein-DNA interactions that assemble the HAF1a complex, during myeloid differentiation (PU.1 is not tyrosine-phosphorylated). However, IFNγ differentiation of U937 cells might also increase total or nuclear abundance of one of these proteins. To determine the contribution of these potential mechanisms to HAF1a binding during U937 differentiation, we investigated abundance, tyrosine phosphorylation, and nuclear localization of the HAF1a complex proteins.

We found that IFNγ treatment of U937 cells does not alter abundance of total or nuclear IRF1 and ICSBP (Fig. 4*A*). Consistent with previous reports (32), IFNγ-induced U937 differentiation increases the amount of total cellular IRF1 and ICSBP that is immunoprecipitated by anti-phosphotyrosine antibody (Fig. 4*B*). Additionally, IFNγ treatment of U937 cells increases nuclear, tyrosine-phosphorylated IRF1 (Fig. 4*C*) and ICSBP (Fig. 4*D*). Consistent with other investigators, we found that PU.1 abundance does not increase during myeloid cell line differentiation and that PU.1 is not tyrosine-phosphorylated in U937 cells, with or without IFNγ treatment (not shown). We also found that CBP is also tyrosine-phosphorylated during U937 differentiation (not shown).

Therefore, our investigations indicate that several component proteins in the HAF1a complex are tyrosine-phosphorylated. Since SHP1-PTP decreases transcriptional activation via the *CYBB* and *NCF2* HAF1 cis elements, we hypothesize that one or more of the HAF1a complex proteins are substrates of either SHP1 or another phosphatase in the SHP1 pathway. To investigate this, we performed anti-phosphotyrosine immunoprecipitation of nuclear proteins from U937 stable transfectants with CS452-SHP1 or vector control. Consistent with our hypothesis, we found that CS453-SHP1 overexpression increases the abundance of tyrosine-phosphorylated IRF1 and ICSBP in undifferentiated U937 cells (Fig. 4*E*). In contrast, CS453-SHP1 overexpression does not increase either total or nuclear IRF1 or ICSBP protein (not shown).

Based upon these results, we further investigated the effect of SHP1 on IRF1 and ICSBP tyrosine phosphorylation. Fortuitously, *in vitro* translated IRF1 and ICSBP are tyrosine phosphorylated (in rabbit reticulocyte lysate). Therefore, we incubated ^{35}S-labeled, *in vitro* translated IRF1 and ICSBP with SHP1 (immunoprecipitated from undifferentiated U937 lysates) or control (irrelevant immunoprecipitate). The *in vitro* translated proteins were subsequently anti-phosphotyrosine immunoprecipitated and separated by SDS-PAGE. We found that incubation with SHP1 (but not control eluate) decreases tyrosine phosphorylation of *in vitro* translated IRF1 and ICSBP (Fig. 4*F*). These results further implicate SHP1 in modulation of IRF1 and ICSBP tyrosine phosphorylation.

Conserved Tyrosine Residues in IRF1 and ICSBP Are Necessary for IFNγ-induced CYBB and NCF2 Transcription—IRF1 and ICSBP share one conserved tyrosine residue in the "IRF" domain, Tyr-109 IRF1 and Tyr-95 ICSBP (34). The IRF domain is hypothesized to be involved in DNA binding and in protein-protein interactions (32, 34). Since these residues are conserved between several IRF proteins, we reasoned that they might be functionally significant. Therefore, we mutated the conserved tyrosine residues in IRF1 and ICSBP to phenylalanine, and we investigated the ability of the mutant proteins to activate transcription via the HAF1 cis elements.

We transfected U937 cells with either an artificial promoter construct containing multiple copies of an HAF1-binding site linked to a minimal promoter and a reporter or minimal promoter control vector (p-cybbhafTATACAT or p-TATACAT control, see above). We co-transfected these cells with plasmids to overexpress various combinations of PU.1, IRF1, Y109F IRF1, ICSBP, and Y95F ICSBP, and we assayed reporter gene ex-

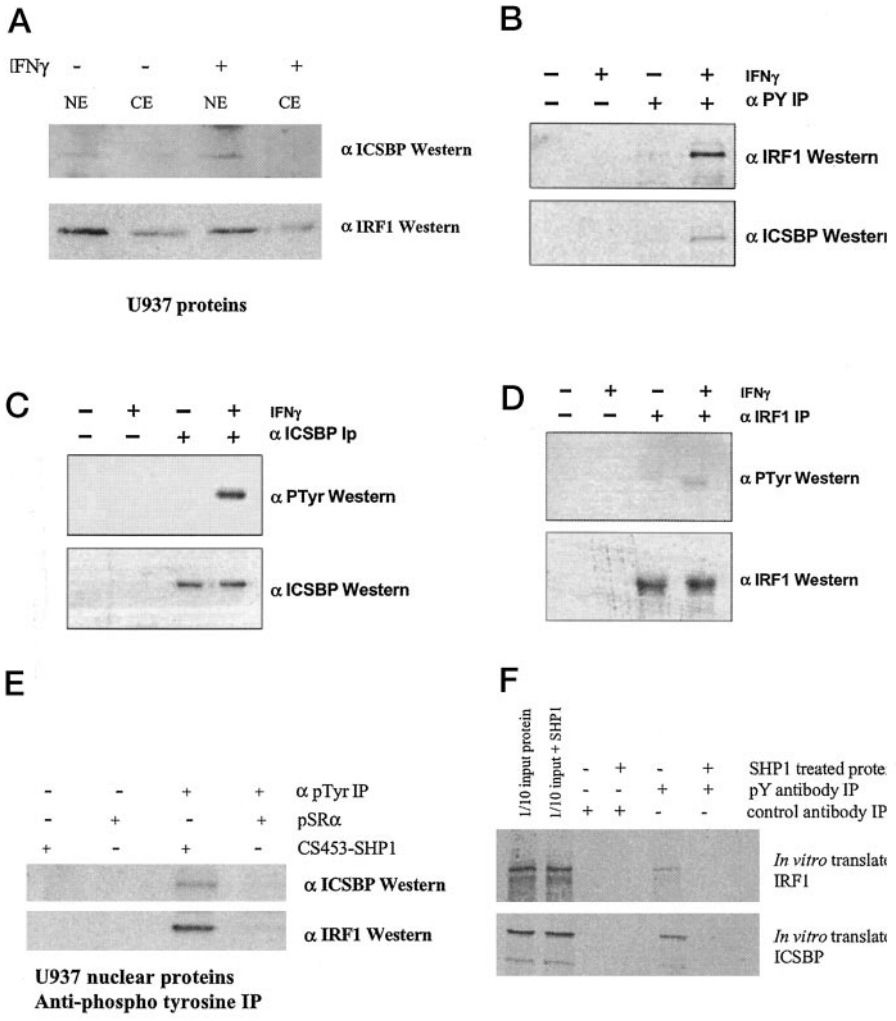

FIG. 4. **IRF1 and ICSBP are tyrosine-phosphorylated during IFNγ-induced U937 differentiation.** *A,* IFNγ differentiation does not alter nuclear *versus* cytoplasmic localization of IRF1 or ICSBP. U937 cells were incubated for 48 h, with or without IFNγ, as indicated. Nuclear and cytoplasmic protein fractions were isolated and separated by SDS-PAGE (30 μg), and Western-blotted proteins were serially probed with antibody to IRF1 and ICSBP as indicated. IFNγ treatment does not significantly alter the abundance of total or nuclear IRF1 or ICSBP. *B,* IFNγ differentiation of U937 cells increases abundance of tyrosine-phosphorylated IRF1 and ICSBP. U937 cells were incubated for 48 h, with or without IFNγ, as indicated. Nuclear proteins (200 μg) were immunoprecipitated with an anti-phosphotyrosine antibody (1.0 μg) or irrelevant, control antibody (mouse anti-rabbit IgG). Immunoprecipitated proteins were analyzed by Western blot with antibodies to IRF1 or ICSBP, as indicated. IFNγ treatment of U937 cells significantly increases the abundance of immunoprecipitable, tyrosine-phosphorylated IRF1 and ICSBP. *C,* IFNγ differentiation of U937 cells increases ICSBP tyrosine phosphorylation but not ICSBP protein abundance. U937 cells were incubated 48 h, with or without IFNγ, as indicated. Nuclear proteins (200 μg) were immunoprecipitated with an anti-ICSBP antibody (1.0 μg) or irrelevant, control antibody (goat anti-rabbit IgG). Immunoprecipitated proteins were analyzed by Western blot with anti-phosphotyrosine or anti-ICSBP antibodies, as indicated. IFNγ treatment of U937 cells significantly increases the abundance of tyrosine-phosphorylated ICSBP but not total ICSBP. *D,* IFNγ differentiation of U937 cells increases IRF1 tyrosine phosphorylation but not IRF1 protein abundance. U937 cells were incubated 48 h with or without IFNγ, as indicated. Nuclear proteins (200 μg) were immunoprecipitated with an anti-IRF1 antibody (1.0 μg) or irrelevant, control antibody (rabbit anti-mouse IgG). Immunoprecipitated proteins were analyzed by Western blot with anti-phosphotyrosine or anti-IRF1 antibodies, as indicated. IFNγ treatment of U937 cells significantly increases the abundance of tyrosine-phosphorylated IRF1 but not total IRF1. *E,* inhibition of SHP1-PTP increases tyrosine phosphorylation of IRF1 and ICSBP in undifferentiated U937 cells. Nuclear proteins were isolated from U937 stable transfectants with CS354-SHP1 or pSRα control and immunoprecipitated (200 μg) with anti-phosphotyrosine antibody (1.0 μg) or irrelevant control antibody (mouse anti-rabbit IgG). Immunoprecipitated proteins were analyzed by Western blot with antibodies to IRF1 or ICSBP, as indicated. Overexpression of CS453-SHP1 in U937 cells increases IRF1 and ICSBP tyrosine phosphorylation. *F,* SHP1-PTP decreases tyrosine phosphorylation of *in vitro* translated IRF1 and ICSBP. U937 lysate proteins (200 μg) were immunoprecipitated with antibody to SHP1 (1.0 μg) or irrelevant antibody control (rabbit anti-mouse IgG). *In vitro* translated, ^{35}S-labeled IRF1 and ICSBP were incubated with either SHP1 or control eluate. The proteins were subsequently immunoprecipitated with anti-phosphotyrosine antibody (1.0 μg) or irrelevant antibody control (rabbit anti-mouse IgG). IRF protein immunoprecipitates were separated by SDS-PAGE and identified by autoradiography, as indicated. Incubation with SHP1 immunoprecipitate decreases tyrosine phosphorylation of IRF1 and ICSBP but does not decrease abundance of these proteins.

pression. We found that functional interaction of Y109F IRF1 and PU.1 is not significantly different than interaction of wild type IRF1 and PU.1 ($n = 14$, $p = 0.745$) (Fig. 5*A*). In contrast, mutation of IRF1 Tyr-109 significantly impairs the ability of IRF1 to interact synergistically with PU.1 and ICSBP to activate the HAF1 cis element in U937 transfectants (59.1% less reporter gene expression in transfectants with Y109F IRF1 *versus* wild type IRF1, $n = 14$, $p < 0.0031$). Indeed, transcriptional activation of p-cybbhafTATACAT by PU.1 + Y109F IRF1 + ICSBP is not significantly different than transcriptional activation by PU.1 + ICSBP ($n = 12$, $p = 0.34$).

We were intrigued to find that mutation of ICSBP Tyr-95 had different functional consequences than mutation of IRF1 Tyr-109. We found that overexpression of Y95F ICSBP and PU.1 does not activate transcription as efficiently as wild type ICSBP and PU.1 (49.0% less, $n = 16$, $p = 0.016$). Indeed, transcriptional activation of p-cybbhafTATACAT by PU.1 + Y95F ICSBP is not significantly different than transcriptional

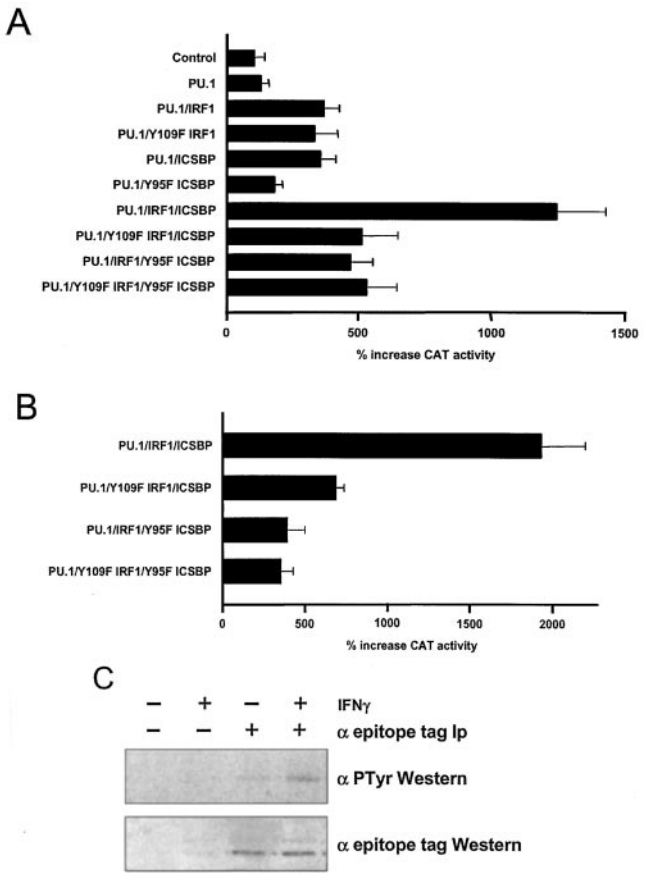

FIG. 5. **Conserved tyrosine residues in IRF1 and ICSBP are necessary for protein-protein interactions that activate *CYBB* and *NCF2* transcription.** *A,* the conserved tyrosine residues in IRF1 (Tyr-109) and ICSBP (Tyr-95) are necessary for activation of the *CYBB* HAF1 cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the *CYBB* HAF1 cis element (p-cybbhafTATACAT), or control (p-TATACAT), and vectors to overexpress various combinations of PU.1, IRF1 or Y109F IRF1, and ICSBP or Y95F ICSBP. Results are expressed as percent increased CAT activity, relative to p-TATACAT vector control. Reporter expression from p-cybbhafTATACAT was not significantly different with overexpression of PU.1 + IRF1 than with PU.1 + Y109F IRF1 ($n = 14$, $p = 0.745$). Additionally, p-cybbhafTATACAT expression was not significantly different with overexpression of PU.1 + Y109F IRF1 + ICSBP than with PU.1 + ICSBP ($n = 12$, $p = 0.34$). In contrast, p-cybbhafTATACAT expression was not significantly different with overexpression of PU.1 than with PU.1 + Y95F ICSBP ($n = 12$, $p = 0.32$). Additionally, p-cybbhafTATACAT expression was not significantly different with overexpression of PU.1 + IRF1 + Y95F ICSBP than with PU.1 + IRF1 ($n = 12$, $p = 0.38$). Consistent with our previous results, these proteins had no effect on p-TATACAT reporter expression. *B,* the conserved tyrosine residues in IRF1 (Tyr-109) and ICSBP (Tyr-95) are necessary for IFNγ-induced activation of the *CYBB* HAF1 cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the *CYBB* HAF1 cis element (p-cybbhafTATACAT), or control (p-TATACAT), and vectors to overexpress various combinations of PU.1, IRF1 or Y109F IRF1, and ICSBP or Y95F ICSBP. Results are expressed as percent increase in CAT activity, relative to p-TATACAT control. Consistent with our previous results, IFNγ differentiation significantly increases p-cybbhafTATACAT expression in U937 transfectants overexpressing PU.1 + IRF1 + ICSBP. In contrast, IFNγ differentiation does not increase p-cybbhafTATACAT expression in U937 transfectants overexpressing PU.1 + IRF1 + Y95F ICSBP, PU.1 + Y109F IRF1 + ICSBP, or PU.1 + Y109F IRF1 + Y95F ICSBP. *C,* overexpressed ICSBP is tyrosine-phosphorylated during IFNγ differentiation of U937 cells. U937 cells, stably transfected with epitope-tagged ICSBP, were incubated 48 h, with or without IFNγ, as indicated. Nuclear proteins (200 μg) were immunoprecipitated with an anti-"xpress" tag antibody (1.0 μg), or irrelevant, control antibody (mouse anti-rabbit IgG). Immunoprecipitated proteins were analyzed by Western blot with anti-phosphotyrosine and anti-"xpress" antibodies, as indicated. IFNγ treatment of U937 cells significantly increases tyrosine phosphorylation of overexpressed ICSBP.

activation of the construct by PU.1 alone ($n = 12$, $p = 0.32$). Additionally, Y95F ICSBP does not functionally cooperate with PU.1 and IRF1 to activate reporter expression via the HAF1 cis element; reporter gene expression with PU.1 + IRF1 + Y95F ICSBP is 62.5% less than with PU.1 + IRF1 + ICSBP ($n = 16$, $p = 0.0003$). Reporter gene expression, via the HAF1 cis element, is not significantly different for transfectants with PU.1 + IRF1 than transfectants with PU.1 + IRF1 + Y95F ICSBP ($n = 12$, $p = 0.38$).

In our previous investigations, IFNγ treatment significantly increased the ability of overexpressed PU.1, IRF1, and ICSBP to activate transcription of artificial promoter constructs with HAF1-binding sites. One possible explanation is that IFNγ induces tyrosine phosphorylation of the overexpressed proteins, increasing affinity of protein-protein-DNA interactions that activate transcription via the HAF1 cis element. To investigate this, we repeated the above experiments with IFNγ-treated U937 transfectants (Fig. 5*B*). We found that IFNγ did not induce a significant increase of p-cybbhafTATACAT reporter expression in transfectants with PU.1, Y109F IRF1, and ICSBP. In contrast, IFNγ induced a significant increase in p-cybbhafTATACAT reporter expression in transfectants with PU.1, IRF1, and ICBP, consistent with our previous results (69.5% less reporter expression in IFNγ treated PU.1, Y019F IRF1, and ICSBP transfectants than with PU.1, IRF1, and ICSBP $n = 4$, $p = 0.0043$). Similarly, mutation of ICSBP Tyr-95 abolished IFNγ-induced cooperative interaction of PU.1, IRF1, and ICSBP with the HAF1 cis element (Fig. 5*B*). Reporter expression from p-cybbhafTATACAT is not significantly different in transfectants with PU.1, IRF1, and Y95F ICSBP, with and without IFNγ treatment (80.0% less in PU.1 + IRF1 + Y95F ICSBP IFNγ-treated transfectants than PU.1 + IRF1 + ICSBP IFNγ-treated transfectants, $n = 4$, $p = 0.00165$).

Our preliminary data indicate that *in vitro* interaction of the HAF1a complex proteins is inhibited by protein-tyrosine phosphatase activity in undifferentiated U937 cells. However, our investigations also indicate that overexpressed PU.1, IRF1, and ICSBP cooperate to activate the HAF1 cis element in undifferentiated U937 transfectants. Additionally, this functional interaction requires the conserved tyrosine residues in IRF1 and ICSBP, in both undifferentiated and differentiated transfectants. These results are consistent with the possibility that tyrosine phosphorylation of IRF1 and ICSBP is not "all or none" but quantitatively increases during IFNγ-induced U937 differentiation and is associated with increased HAF1a complex binding. Therefore, overexpressed IRF1 and ICSBP may be tyrosine-phosphorylated in undifferentiated U937 cells, but the amount of tyrosine phosphorylation may increase in IFNγ-differentiated cells. Since PTP activity decreases transcription via the HAF1 cis elements in undifferentiated U947 cells, overexpressed IRF1 and ICSBP may overwhelm the capacity of endogenous PTPs. Since IFNγ decreases SHP1-PTP activity and increases PTK activity, differentiation of the transfectants would increase tyrosine phosphorylation of the overexpressed IRF proteins.

To address this possibility, we investigated tyrosine phosphorylation of overexpressed IRF1 and ICSBP in U937 cells, with and without IFNγ. We selected stable transfectants of U937 cells, overexpressing epitope-tagged ICSBP or IRF1 (or empty vector control). By using the "xpress" epitope tag, we immunoprecipitated overexpressed IRF proteins from U937 lysates (under conditions that do not co-immunoprecipitate other proteins), and we analyzed the immunoprecipitate by anti-phosphotyrosine and anti-tag Western blot (Fig. 5*C*). We found that overexpressed ICSBP and IRF1 are minimally tyrosine-phosphorylated and that tyrosine phosphorylation is

significantly increased by IFNγ-induced differentiation of the cells (data shown for ICSBP). These results suggest that IFNγ treatment of U937 transfectants increases IRF1 and ICSBP tyrosine phosphorylation and that tyrosine phosphorylation of the conserved tyrosine residues in these IRF proteins is associated with increased interaction with the HAF1 cis element.

The Conserved Tyrosine Residues in IRF1 and ICSBP Are Necessary for HAF1a Complex Protein-Protein Interactions—We next investigated the hypothesis that conserved tyrosine residues in IRF1 and ICSBP increase the affinity of these proteins for interaction with each other or with PU.1. For these experiments, IRF1, Y109F IRF1, ICSBP, and Y95F ICSBP were translated *in vitro* in rabbit reticulocyte lysate (5). In control experiments, anti-phosphotyrosine antibody immunoprecipitates *in vitro* translated IRF1, Y109F IRF1, ICSBP and Y95F ICSBP (IRF1 has 8 tyrosine residues and ICSBP has 12). We used these proteins to investigate whether Tyr-109 IRF1 or Tyr-95 ICSBP is necessary for interaction with PU.1.

We expressed PU.1 as a GST fusion protein in *E. coli* and affinity purified the protein, as described previously (5). To serine-phosphorylate PU.1, the affinity-purified protein was incubated in rabbit reticulocyte lysate, as described previously (22). We found that the affinity of PU.1/GST for *in vitro* translated IRF1 is not different than the affinity of PU.1/GST for Y109F IRF1 (Fig. 6*A*). Additionally, we found that interaction of PU.1 with IRF1 is not increased by the presence of *in vitro* translated ICSBP (ICSBP is unlabeled in these binding reactions, since IRF1 and ICSBP migrate with similar mobility). In contrast, we found that the affinity of PU.1/GST for Y95F ICSBP is less than the affinity of PU.1/GST for wild type ICSBP. This result is consistent with our transfection data above. Additionally, the affinity of PU.1/GST for ICSBP is slightly increased by the presence of wild type IRF1 in the binding reactions (IRF1 is unlabeled in these binding reactions) (Fig. 6*B*).

We also tested the affinity of IRF1/GST and Y019F IRF1/GST for *in vitro* translated ICSBP and Y95F ICSBP (Fig. 6*C*). In these experiments, IRF1/GST fusion proteins were tyrosine-phosphorylated by preincubation in rabbit reticulocyte lysate. We found that the affinity of IRF1/GST binding to ICSBP is higher than IRF1/GST binding to Y95F ICSBP. Additionally, Y109F IRF1/GST does not interact with either ICSBP or Y95F ICSBP. In these experiments, we noted that the affinity of PU.1/GST for ICSBP is greater than the affinity of IRF1/GST for ICSBP.

However, it is possible that phosphorylation of these proteins is not the same in myeloid cells as in rabbit reticulocyte lysate. Therefore, we overexpressed mutant and wild type IRF1 and ICSBP in U937 cells, and we investigated the impact of conserved tyrosine residue mutation on assembly of the HAF1a complex by EMSA. Stable U937 transfectants were generated, overexpressing epitope-tagged IRF1, Y109F IRF1, ICSBP, or Y95F ICSBP (see above). Nuclear proteins were isolated from IFNγ-differentiated U937 transfectants, and overexpression of the IRF fusion proteins was documented by anti-epitope tag Western blot (not shown). These blots indicated that wild type IRF and conserved tyrosine mutant IRF proteins were equivalently overexpressed. Therefore, we performed EMSA with a HAF1 cis element probe and nuclear proteins from these U937 stable transfectants.

In EMSA with nuclear proteins from either IRF1- or ICSBP-overexpressing U937 cells, anti-epitope antibody partly disrupts binding of the HAF1 complex and completely disrupts binding of the HAF1a complex (Fig. 6*D*). This is consistent with our previous results (6) with antibodies to endogenous IRF1 or ICSBP. However, in EMSA with nuclear proteins from Y109F IRF1-overexpressing cells, anti-epitope antibody recognizes only the HAF1 complex (Fig. 6*D*). In contrast, anti-epitope antibody does not recognize either the HAF1 or HAF1a complex, in EMSA with nuclear proteins from Y95F ICSBP-overexpressing cells (Fig. 6*D*). In control EMSA with nuclear proteins from empty vector U937 transfectants, epitope tag antibody did not recognize any complex interacting with the HAF1 probe.

Therefore, the results of these two different protein-protein interaction assays are consistent. Our investigations indicate that mutation of IRF1 Tyr-109 does not impair interaction with PU.1 but decreases the affinity of IRF1 interaction with ICSBP. Therefore, Y109F IRF1 participates in the HAF1 but not the HAF1a complex. In contrast, these experiments suggest that mutation of ICSBP Y95 decreases the affinity of interaction with both PU.1 and IRF1. Therefore, Y95F ICSBP does not participate in either the HAF1 or HAF1a complex. These results are also consistent with our transfection data presented above.

DISCUSSION

During myeloid differentiation, expression of gp91^{PHOX} and p67^{PHOX} is the rate-limiting step for respiratory burst oxidase activity (33). Previously, we determined that HAF1 cis elements in the *CYBB* and *NCF2* genes are necessary for lineage-specific and differentiation stage-specific gp91^{PHOX} and p67^{PHOX} expression. We found that these cis elements are activated by cooperation between the transcription factors PU.1, IRF1, ICSBP, and CBP. Since expression of PU.1 and ICSBP is restricted to myeloid and B-cells, our previous investigations (5, 6) identified a mechanism of lineage restriction of *CYBB* and *NCF2* transcription. The current studies identify a mechanism of differentiation stage restriction of transcriptional activation by PU.1, IRF1, ICSBP, and CBP.

Our investigations indicate that SHP1-PTP activity inhibits *CYBB* and *NCF2* transcription in undifferentiated myeloid cells lines. Additionally, we determined that SHP1-PTP activity decreases IRF1 and ICSBP tyrosine phosphorylation, and interaction of the PU.1/IRF1/ICSBP/CBP complex with *CYBB* and *NCF2* HAF1 cis elements. Consistent with this, we also found that activation of the HAF1 cis elements requires specific IRF1 and ICSBP tyrosine residues. Therefore, our investigations suggest that the SHP1-PTP signaling pathway antagonizes myeloid differentiation and phagocyte function by decreasing tyrosine phosphorylation of interferon regulatory factors. These investigations also indicate that differentiation stage-specific myeloid gene transcription is dependent upon cytokine-induced, differentiation stage-specific, post-translational modification of interferon regulatory factors.

In our investigations, either dominant negative SHP1 (CS453) overexpression or IFNγ differentiation induces approximately equivalent decrease in SHP1-PTP activity in U937 cells. Consistent with this, either CS453-SHP1 overexpression or IFNγ treatment of U937 cells induces equivalent activation of an artificial promoter construct with multiple copies of the *CYBB* HAF1 cis element. These results suggest that the SHP1-PTP activity plays a major role in regulating activity of the HAF1 cis elements during myeloid differentiation. However, CS453-SHP1 overexpression induces less than a 2-fold increase in activity of reporter constructs with 470 bp of *CYBB* promoter or 400 bp of *NCF2* 5′ flank in U937 cells, although IFNγ differentiation increases expression of these constructs 4- and 5-fold, respectively (6). These results suggest that the *CYBB* and *NCF2* genes also include cis elements that enhance transcription in an SHP1-PTP-independent manner.

Consistent with these transfection experiments, equivalent *in vitro* HAF1a complex binding is induced by either IFNγ

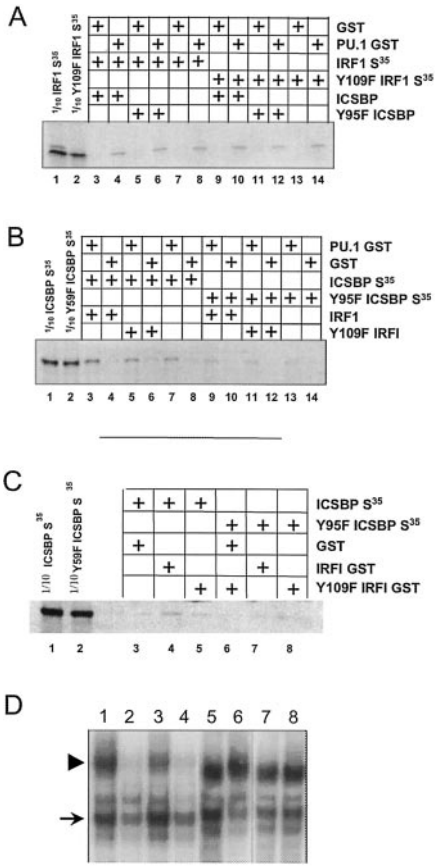

FIG. 6. **Conserved tyrosine residues in IRF1 and ICSBP increase the affinity of protein-protein interactions.** *A,* PU.1 has equivalent affinity for IRF1 and Y109F IRF1. PU.1/GST and control GST were analyzed for binding of *in vitro* translated, [^{35}S]methionine-labeled IRF1 or Y109F IRF1. Binding assays were also incubated with either control reticulocyte lysate, *in vitro* translated unlabeled ICSBP, or Y95F ICSBP, as indicated. Precipitating proteins were separated by SDS-PAGE, and co-precipitation of IRF1 or Y109F IRF1 with PU.1/GST or GST was detected by autoradiography. IRF1 and Y109F IRF1 bind PU.1/GST (but not control GST) with equal affinity, and binding affinity is not altered by ICSBP. *B,* PU.1 has greater affinity for ICSBP than for Y95F ICSBP. PU.1/GST and control GST were analyzed for binding of *in vitro* translated, [^{35}S]methionine-labeled ICSBP or Y95F ICSBP. Binding assays were incubated with either control reticulocyte lysate, *in vitro* translated, unlabeled IRF1, or Y109F IRF1, as indicated. Precipitating proteins were separated by SDS-PAGE, and co-precipitation of ICSBP or Y95F ICSBP with PU.1/GST or GST was detected by autoradiography. ICSBP binds PU.1/GST (but not control GST) with greater affinity than Y95F ICSBP. Binding affinity of both ICSBP and Y95F ICSBP for PU.1/GST is slightly increased by IRF1 but not Y109F IRF1. *C,* IRF1 has greater affinity for ICSBP than for Y95F ICSBP, and Tyr-109 mutant IRF1 has less affinity for ICSBP than does wild type IRF1. IRF1/GST, Y109F IRF1/GST, and control GST were analyzed for binding of *in vitro* translated, [^{35}S]methionine-labeled ICSBP or Y95F ICSBP, as indicated. Precipitating proteins were separated by SDS-PAGE, and co-precipitation of ICSBP or Y95F ICSBP with IRF1/GST, Y109F IRF1/GST, or GST was detected by autoradiography. ICSBP binds IRF1/GST with greater affinity than Y95F ICSBP, and neither *in vitro* translated protein co-precipitates efficiently with Y109F IRF1/GST. *D,* Y109F IRF1 does not participate in the HAF1 complex binding the *CYBB* HAF1 cis element, and Y95F ICSBP does not participate in either the HAF1 or HAF1a complex. U937 cells were transfected with a vector to overexpress epitope-tagged IRF1 (*lanes 1* and *2*), ICSBP (*lanes 3* and *4*), Y109F IRF1 (*lanes 5* and *6*), or Y95F ICSBP (*lanes 7* and *8*). Transfectants were treated with IFNγ for 48 h, and nuclear proteins were used in EMSA with the *CYBB* HAF1 cis element probe. Binding reactions were preincubated with either anti-"xpress" epitope antibody (*lanes 2, 4, 6,* and *8*) or irrelevant, control antibody (*lanes 1, 3, 5,* and *7*). The HAF1 complex is indicated by the *arrow* and the HAF1a complex by the *arrowhead.* Epitope-tagged IRF1 and ICSBP are present in the HAF1 and HAF1a complexes, consistent with our previous results with endogenous proteins. However, Y109F IRF1 is only present in the HAF1 complex, and Y95F ICSBP is not present in either the HAF1 or HAF1a complex.

differentiation or CS453-SHP1 overexpression in U937 cells. Additionally, both CS453-SHP1 overexpression and IFNγ differentiation induce IRF1 and ICSBP tyrosine phosphorylation. Similarly, SHP1 decreases *in vitro* HAF1a binding and IRF1 and ICSBP tyrosine phosphorylation. These results indicate that IRF tyrosine phosphorylation correlates with HAF1a complex binding affinity. Furthermore, endogenous SHP1, IRF1, and ICSBP co-localize in the nuclear fraction of undifferentiated U937 lysates, providing an additional connection between SHP1-PTP activity and IRF1 and ICSBP function. Our results suggest that IRF1 and ICSBP may be SHP1-PTP substrates. However, it is possible that an unidentified PTP, present in substrate proteins (rabbit reticulocyte lysate or nuclear proteins from IFNγ-differentiated U937 cells), is activated by SHP1-PTP and de-phosphorylates IRF1 and ICSBP. Nevertheless, this possibility also implicates SHP1-PTP activity in regulation of IRF protein-tyrosine phosphorylation state in undifferentiated myeloid cells. Therefore, our investigations provide a link between the IFNγ receptor, SHP1-PTP, the IRF transcription factor family, and differentiation stage-specific myeloid gene transcription.

Although SHP1-PTP activity disrupts *in vitro* HAF1a complex binding, SHP1-PTP activity does not influence *in vitro* HAF1 complex binding (PU.1/IRF1 or PU.1/ICSBP). Our investigations with IRF1 and ICSBP tyrosine mutants indicate a potential mechanism for this. We found that Y109F IRF1 interacts with PU.1 but does not cooperate with PU.1 + ICSBP in either transfection experiments or *in vitro* binding assays. In contrast, Y95F ICSBP interacts with neither PU.1 nor PU.1 + IRF1 in transfection or *in vitro* binding assays. These results suggest that the HAF1 complex includes PU.1 + IRF1 in undifferentiated, non-PTP inhibitor-treated cells. Our investigations also suggest that phosphorylation of the conserved tyrosine residues in IRF1 and ICSBP increases the affinity of different, functionally significant protein-protein interactions. Although these results imply that the conserved tyrosine residues in IRF1 and ICSBP are phosphorylated during IFNγ-induced U937 differentiation, we have not demonstrated this directly. Identification of IRF1 and ICSBP tyrosine residues that are phosphorylated in response to cytokine-induced myeloid differentiation are planned. Such investigations will permit identification of differentiation stage-specific, phosphorylation dependent protein-protein interactions that are involved in myeloid gene transcription.

Therefore, the current investigations provide insight into functional regulation of IRF proteins. Our results suggest a mechanism by which ICSBP mediates differentiation stage-specific gene expression at multiple points during myelopoiesis. In immature myeloid cells, non-tyrosine-phosphorylated ICSBP represses transcription by binding directly to negative cis elements (32, 34). In contrast, we found that tyrosine-phosphorylated ICSBP activates *CYBB* and *NCF2* transcription, during late myeloid differentiation, by interacting with PU.1, IRF1, and CBP. Similar ICSBP protein-protein interactions are necessary for activation of the *TOLL-like receptor 4* gene in mature monocytes (22). Therefore, cytokine-dependent tyrosine phosphorylation may alter ICSBP function during myeloid differentiation. Similarly, tyrosine phosphorylation may be a mechanism by which the ubiquitous IRF1 regulates gene transcription in response to inflammatory cytokines.

IFNγ treatment of myeloid cell lines activates JAK1 and 2 protein tyrosine kinases (PTK), leading to transcription factor phosphorylation, cellular differentiation, and activation. Our investigations indicate that IFNγ differentiation also decreases SHP1-PTP activity. Therefore, we hypothesize that a shift between specific PTP and PTK activities occurs during myeloid

differentiation. Our data suggest that this balance dictates the phosphorylation state of transcription factors involved in stage-specific myeloid gene transcription. Since mature phagocytes are activated by IFNγ, the balance between kinase and phosphatase activity may also be altered during the inflammatory response, providing a mechanism for reversible oxidase gene expression. Since U937 cells are differentiated and primed for activation by IFNγ, our results are consistent with SHP1-PTP regulation of oxidase gene transcription during myelopoiesis, the inflammatory response, or both.

Therefore, these investigations implicate SHP1-PTP signaling in regulation of IRF1 and ICSBP tyrosine phosphorylation state, and IRF tyrosine phosphorylation in activation of oxidase gene transcription. However, SHP1 may have multiple effects on transcription factor tyrosine phosphorylation. For example, SHP1-PTP may inactivate protein tyrosine kinases that are involved in IRF phosphorylation. Therefore, our investigations indicate that identifying intermediates in the IFNγ/SHP1-PTP/IRF pathway is important in understanding molecular events that regulate myeloid differentiation and the inflammatory response.

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SHP1 Protein-tyrosine Phosphatase Inhibits gp91^{PHOX} and p67^{PHOX} Expression by Inhibiting Interaction of PU.1, IRF1, Interferon Consensus Sequence-binding Protein, and CREB-binding Protein with Homologous Cis Elements in the *CYBB* and *NCF2* Genes

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