Activation of SHP2 Protein-tyrosine Phosphatase Increases HoxA10-induced Repression of the Genes Encoding gp91\textsuperscript{PHOX} and p67\textsuperscript{PHOX}\textsuperscript{*}

The CYBB and NCF2 genes encode the phagocyte oxidase proteins gp91\textsuperscript{PHOX} and p67\textsuperscript{PHOX}, respectively. These genes are transcribed after the promyelocyte stage of differentiation, and transcription continues until cell death. In undifferentiated myeloid cells, homologous cis-elements in the CYBB and NCF2 genes are repressed by the homeodomain transcription factor HoxA10. During cytokine-induced myelopoiesis, tyrosine phosphorylation of HoxA10 decreases binding affinity for the CYBB and NCF2 cis-elements. This abrogates HoxA10-induced transcriptional repression as differentiation proceeds. Therefore, mechanisms involved in differentiation stage-specific HoxA10 tyrosine phosphorylation are of interest because HoxA10 phosphorylation modulates myeloid-specific gene transcription. In this study, we found that HoxA10 is a substrate for SHP2 protein-tyrosine phosphatase in undifferentiated myeloid cells. In contrast, HoxA10 is a substrate for a constitutively active mutant form of SHP2 in both undifferentiated and differentiating myeloid cells. Expression of such SHP2 mutants results in persistent HoxA10 repression of CYBB and NCF2 transcription during myelopoiesis. Both HoxA10 overexpression and activating SHP2 mutations have been described in human myeloid malignancies. Therefore, our results suggest that these mutations could cooperate, leading to decreased myeloid-specific gene transcription and functional differentiation block in myeloid cells with both defects.

The murine and human HOX genes are divided into four paralog groups (A–D) on four different chromosomes. These genes encode highly conserved homeodomain transcription factors that are expressed cephalad to caudal during embryogenesis (1). Additionally, HOX gene transcription is differentiation stage-specific during definitive hematopoiesis (2). For example, the HOX1–4 genes are maximally expressed in hematopoietic stem cells (3). In contrast, HoxA5–13 (also referred to as the ABD HOXA genes) are maximally expressed in committed myeloid progenitors (3). Differentiation stage-specific expression suggests that Hox proteins may regulate progression of myelopoiesis. Indeed, a number of studies indicate the importance of HoxA proteins for normal myeloid development (5–9).

The function of Abd HoxA proteins has been studied in murine gene “knock-out” experiments. These studies found no profound hematopoietic phenotype in Hoxa7, Hoxa9, or Hoxa10-deficient mice (10–12). This result suggests possible functional redundancy between these proteins. In contrast, overexpression of various Abd HoxA proteins results in significant abnormalities in murine bone marrow transplantation experiments. For example, mice transplanted with HoxA10-overexpressing bone marrow rapidly develop leukocytosis, characterized by circulating mature phagocytes (7). This myeloproliferative disorder evolves to clonal acute myeloid leukemia (AML)\textsuperscript{2} over several months. Therefore, HoxA10 overexpression does not induce differentiation block and therefore is not adequate to induce AML. In this model, the time lag between myeloproliferation and AML is hypothesized to permit accumulation of additional genetic mutations; however, such cooperating mutations have not been identified.

A study of bone marrow samples from human subjects with AML also suggests a role for HoxA10 overexpression in leukemogenesis (1). Expression of the entire group of Abd HoxA proteins has been demonstrated in human AML bone marrow samples from subjects with translocations involving the MLL (mixed lineage leukemia) gene (8). In murine transplantation experiments, expression of such leukemia-associated MLL fusion proteins induces myeloproliferation, which progresses to blast crisis over several months (14–19). Although such studies suggest that Abd HoxA proteins play important roles in normal and malignant hematopoiesis, relatively few genuine HoxA target genes have been identified. In previous studies, we found that HoxA10 represses transcription of the genes encoding two phagocyte respiratory burst oxidase proteins: gp91\textsuperscript{PHOX} and p67\textsuperscript{PHOX} (the CYBB and NCF2 genes, respectively) (20, 21). These genes are actively transcribed after the promyelocyte stage of myelopoiesis, and transcription continues until cell death (22, 23).

We found that CYBB and NCF2 transcription is repressed in undifferentiated myeloid cells by interaction of HoxA10, Pbx1, and HDAC2 with homologous cis-elements in these genes (24).

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\textsuperscript{2} To whom correspondence should be addressed: Feinberg School of Medicine, Olson Pavilion, Rm. 8524, 710 North Fairbanks Ct., Chicago, IL 60611. Tel.: 312-503-4625; Fax: 312-908-5717; E-mail: e-eklund@northwestern.edu.
Pbx1 is dispensable for repression activity, which involves direct interaction of HDAC2 with a novel HoxA10 repression domain. Pbx1 is hypothesized to increase the DNA binding affinity of Hox proteins in such circumstances (25). Additional studies suggest that HoxA10 also represses transcription of the genes encoding lactoferrin and neutrophil collagenase in undifferentiated myeloid cells (26, 27). Therefore, HoxA10 represses transcription of multiple genes that confer the mature myeloid phenotype.

HoxA10 binding to the CYBB and NCF2 cis-elements decreases during myeloid differentiation in vitro and in vivo (20, 21). However, HoxA10 protein abundance is relatively constant during differentiation of committed myeloid progenitor cells. In a previous study, we found that HoxA10 is tyrosine-phosphorylated during cytokine-induced myelopoiesis (28). We also found that phosphorylation of two tyrosine residues in the HoxA10 homeodomain decreases binding affinity for the CYBB and NCF2 promoters (21, 28). Therefore, cytokine-induced post-translational modification regulates HoxA10 repression of myeloid-specific gene transcription.

These results have implications for the role of HoxA10 in leukemogenesis. Specifically, HoxA10 overexpression might be expected to block transcription of genes conferring the mature myeloid phenotype, resulting in differentiation block and blast crisis. However, overexpressed HoxA10 does not repress transcription of such genes because it is tyrosine-phosphorylated in response to hematopoietic cytokines (20, 21, 28). Therefore, differentiation block by overexpressed HoxA10 requires inhibition of cytokine-induced tyrosine phosphorylation. This could be due to decreased kinase activity or increased phosphatase activity. The time to acquire such genetic lesions might explain the lag between HoxA10-induced myeloproliferation and development of AML in the murine model.

Although the HoxA10 kinase has not been identified, we found that Jak2 activation is sufficient for HoxA10 tyrosine phosphorylation (29). Additionally, we determined previously that SHP1 protein-tyrosine phosphatase (PTP) is involved in maintaining HoxA10 in a non-tyrosine-phosphorylated state in immature myeloid cells (28). During cytokine-induced differentiation, SHP1 PTP activity decreases, increasing HoxA10 tyrosine phosphorylation. However, leukemia-associated activating mutants of SHP1 have not been identified.

In contrast, activating mutations in the closely related SHP2 PTP have been identified in samples from human subjects with myelodysplastic syndromes, AML, or juvenile chronic myelomonocytic leukemia (30, 31). In the resting state, the catalytic domain in SHP2 is masked. Activation occurs when phosphotyrosine residues in a substrate or another protein interact with an SH2 domain (Src homology 2) domain in SHP2, resulting in a conformational change. Previously described leukemia-associated mutations induce a conformational change that unmaskes the catalytic domain, resulting in constitutive activation (32). Mice transplanted with bone marrow expressing such SHP2 PTP mutants develop a myeloproliferative disorder, but not AML (33, 34).

In this study, we investigated whether HoxA10 is a substrate for leukemia-associated activating SHP2 mutants in differentiating myeloid cells. We also investigated the impact of expressing such SHP2 mutants on repression of CYBB and NCF2 transcription by overexpressed HoxA10 as a model for disease progression in myeloid malignancy. The goal of this study was to determine whether HoxA10 overexpression and activating SHP2 PTP mutants might be cooperating genetic defects in the multistep process leading from myeloproliferation to acute myeloid leukemia.

**MATERIALS AND METHODS**

**Plasmids and PCR Mutagenesis**

**Protein Expression Vectors**—The human HoxA10 cDNA was obtained from Dr. C. Largman (University of California, San Francisco) (35). This cDNA sequence represents the major transcript in mammalian hematopoietic cells, encoding a 393-amino acid 55-kDa protein. HoxA10 cDNA sequence was subcloned into the pcDNAamp vector for *in vitro* translation, pSRα for expression in mammalian cells (36), and pMSCV (Stratagene, La Jolla, CA) for expression in murine bone marrow cells. The cDNA for SHP2 PTP was obtained from Dr. Stuart Frank (University of Alabama, Birmingham, AL). A leukemia-associated activating SHP2 mutant (E76K) was generated by site-directed mutagenesis using the Stratagene QuickChange protocol as described (37). Mutant clones were sequenced on both strands to verify that only the intended mutations had been introduced. Wild-type and E76K SHP2 were subcloned into the pMSCVneo vector (Stratagene) for retroviral production. These cDNAs were also subcloned into the pcDNAamp vector for *in vitro* protein translation. Wild-type SHP1 and SHP2 were subcloned into the pGEX1 vector (Amersham Biosciences) for expression in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST).

**Reporter Constructs**—Artificial promoter-reporter constructs were generated in the minimal promoter-reporter vector pTATACAT (obtained from Dr. Andrew Kraft, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC) as described previously (38). A construct was generated with four copies of the negative cis-elements repressed by HoxA10 from the CYBB (−94 to −134 bp) or NCF2 (−600 to −637 bp) promoter subcloned into this vector in the forward direction (referred to as p-cybbTATACAT and p-nclf2TATACAT, respectively) (20, 21).

**Oligonucleotides**

Oligonucleotides were synthesized by the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University as follows: dscybbA10 (the Hox/Pbx-binding sequence from the CYBB promoter, −94 to −134 bp), 5’tcaggtttcgttcagattttagaaccttttctagaat−3’; and dsncf2A10 (a homologous sequence from the NCF2 promoter, −600 to −637 bp). In these oligonucleotides, the HoxA10 core is in boldface, the Pbx core is in *italics*, and ccaat boxes are *underlined*. Complementary single-stranded oligonucleotides were annealed and used in electrophoretic mobility shift assays (EMSAs) or subcloned to generate artificial promoter constructs as described (20, 21).
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Myeloid Cell Line Culture

The human myelomonocytic cell line U937 (39) was obtained from Dr. Andrew Kraft. Cells were maintained and differentiated as described (20, 21, 28). For differentiation experiments, U937 cells were treated for 48 h with 500 units/ml human recombinant interferon-γ (IFN-γ) (Roche Applied Science) (20).

Transfections and Reporter Gene Assays

Transfections for Promoter Analysis—U937 cells were cultured and transfected as described previously (20, 21). Cells (32 × 10^6/sample) were transfected with vector expressing HoxA10 (pSRα/HoxA10) or empty control vector; wild-type SHP2 (pSX/SHP2), SHP2 with a leukemia-associated activating mutation (pSX/E76K SHP2), a dominant-negative form of SHP2 (pSX/SHP2), SHP2 with a leukemia-associated activating mutation (pSX/E76K SHP2), or empty control vector; the minimal promoter-reporter vector pTATACAT with four copies of the CYBB -94 to -134 bp sequence (p-cybbTATACAT), four copies of the NCF2 -600 to -637 bp sequence (p-ncf2TATACAT), or the empty pTATACAT control vector; and pCMV-β-galactosidase (to control for transfection efficiency). Transfectants were harvested 48 h after transfection with and without incubation with human recombinant IFN-γ (500 units/ml). Lysates were analyzed for chloramphenicol acetyltransferase and β-galactosidase activities as described (20).

Transfections for Stable Pools Overexpressing SHP2—U937 cells were transfected with empty expression vector or vector overexpressing wild-type or E76K SHP2 as described above. After 24 h, the medium was supplemented with G418 to 1 mg/ml, and stable transfectant pools were selected over 7–14 days as described previously (20). Stable transfectant pools were selected instead of clones to compensate for possible integration site effects. All experiments were repeated with at least two independent transfectant pools for each construct.

Murine Bone Marrow Culture and Transduction

Bone marrow mononuclear cells were obtained from the femurs of wild-type mice. Bipotential myeloid progenitor cells were cultured (at a concentration of 2 × 10^6 cells/ml) for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN), and 5 ng/ml murine recombinant IL-3 (R&D Systems). After retroviral transduction (see below), cells were either maintained in GM-CSF and interleukin-3 (IL-3; murine progenitor cells) or switched to Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 10 ng/ml murine recombinant macrophage colony-stimulating factor (M-CSF; R&D Systems) for 96 h (monocyte differentiation). Cells were harvested, and cell lysates were used in Western blot experiments as described below.

Retroviral Transduction of Murine Bone Marrow Myeloid Cells

High titer murine stem cell retroviral supernatants were produced using the pMSCVneo vector and the PT67 cell line following the instructions of Stratagene. Filtered retroviral supernatants were used immediately or stored at -80 °C. Transductions of murine bone marrow myeloid progenitor cells were performed as described previously (37). Briefly, cells were harvested, and 4.0 × 10^7 cells were plated in 3 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10 ng/ml GM-CSF, and 5 ng/ml IL-3. An equal volume of retroviral supernatant was added to each dish, and Polybrene was added to a final concentration of 6 μg/ml. Cells were incubated for 8 h at 37 °C and 5% CO2 and then diluted 3-fold with medium supplemented as described above. Cells were incubated overnight, and the procedure was repeated the next day. The day after transduction, G418 was added to 250 ng/ml. Cells were selected in antibiotics for 48 h and then treated with cytokines as indicated. Each experiment was repeated at least three times. Expression of transduced proteins was independently verified by Western blotting for each experiment.

Isolation of Nuclear Proteins and EMSAs

Nuclear extract proteins were isolated from U937 cells by the method of Dignam et al. (40) with protease inhibitors as described (41). In some experiments, U937 cells were differentiated with 500 units/ml IFN-γ before nuclear protein isolation. Oligonucleotide probes were prepared, and EMSAs and antibody supershift assays were performed as described (20, 21). Rabbit anti-HoxA10 polyclonal antibody was generated against a unique peptide (Covance, Inc.) and has been described previously (20, 21, 28). Irrelevant control anti-GST antibody was obtained from Santa Cruz Biotechnology, Inc.

Chromatin Immunoprecipitation

U937 cells were cultured with or without IFN-γ for 48 h as described above. Cells for chromatin immunoprecipitation were incubated with formaldehyde prior to lysis, and lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb as described (42). Lysates underwent immunoprecipitation with either antiserum to HoxA10 or preimmune serum as described (42). Coprecipitated chromatin was analyzed by PCR for HoxA10-specific antibody coprecipitation of the CYBB and NCF2 gene promoters. For these experiments, input chromatin was used as a positive control, and chromatin precipitated by preimmune serum was used as a negative control. PCR products were analyzed by acrylamide gel electrophoresis. The identity of the PCR product was verified by subcloning into a plasmid vector, followed by dideoxy sequencing.

Immunoprecipitation and Western Blotting

Western Blotting of Lysate Proteins from Murine Bone Marrow Cells—Murine bone marrow cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose according to standard techniques. Western blots were serially probed with antibodies to gp91^PHOX, p67^PHOX, SHP2, HoxA10, and glyceraldehyde-3-phosphate dehydrogenase (to control for loading).

Anti-phosphotyrosine Immunoprecipitation and Western Blotting—U937 cells were lysed, and lysates were immunoprecipitated under denaturing conditions with HoxA10 antiserum or preimmune serum control. Murine bone marrow-derived myeloid cells were lysed and immunoprecipitated under dena-
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turing conditions with antibody to phosphotyrosine (clone 4G10, Upstate, Charlottesville, VA) or irrelevant control antibody (anti-GST tag) as described previously (28). Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose as described above. Western blots of U937 proteins were serially probed with antibodies to phosphotyrosine and HoxA10. Blots of murine myeloid cell proteins were probed with HoxA10-specific antibody.

**In Vitro Protein Translation and Phosphatase Assay**

*In vitro* transcribed HoxA10 and E76K SHP2 mRNAs were generated from linearized template DNA using the Riboprobe system (Promega Corp.) according to the manufacturer’s instructions. *In vitro* translated proteins were generated in rabbit reticulocyte lysate (Promega Corp.) according to the manufacturer’s instructions. Control (unprogrammed) lysates were generated in similar reactions in the absence of input RNA. For these experiments, HoxA10 (but not E76K SHP2) was radiolabeled by including [35S]methionine in the translation reaction. For dephosphorylation assays, *in vitro* translated HoxA10 was incubated for 30 min at 30 °C with either *in vitro* translated E76K SHP2 or control lysates. The HoxA10 tyrosine phosphorylation state was determined by immunoprecipitating the reaction with anti-phosphotyrosine or irrelevant control antibody under denaturing conditions as described previously (28). Briefly, precipitated proteins were separated by SDS-PAGE followed by autoradiography to detect *in vitro* translated HoxA10 that coprecipitated with anti-phosphotyrosine antibody.

In other experiments, endogenous HoxA10 was immunoprecipitated from U937 nuclear proteins (300 μg) using antiserum to HoxA10 versus preimmune serum control under denaturing conditions. Proteins were recovered with staph protein A-Sepharose beads and washed several times with radiomune precipitation assay buffer. Precipitated proteins were incubated with *in vitro* translated E76K SHP2 or phosphatase-inactive C463S SHP2 (as a negative control) as described above. *In vitro* translated proteins were generated unlabeled in rabbit reticulocyte lysate as described above. After incubation, the staph protein A beads were washed several times with radiomune precipitation assay buffer, and proteins were separated by SDS-PAGE. Western blots were serially probed with antibodies to phosphotyrosine and HoxA10.

**GST Fusion Protein Pulldown Assays**

*E. coli* JM109 cells transformed with SHP1 or SHP2 in the pGEX vector (or empty control vector) were grown to log phase, supplemented with 0.1 mM isopropyl β-D-thiogalactopyranoside, 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 MgCl2, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 5 mM NaF) and sonicated on ice. Debris was removed by centrifugation, and the lysate was incubated for 30 min 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 *in vitro* translated [35S]methionine-labeled HoxA10 protein and washed extensively with HN buffer. Proteins were eluted with SDS-PAGE sample buffer and separated by 15% SDS-PAGE, and autoradiography was performed.

**RNA Isolation and Quantitative Real-time PCR**

Total cellular RNA was isolated as described previously (43). Reverse transcription reactions were performed using the ImProm-II reverse transcriptase kit (Promega Corp.) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed using the ABI 7900 system (Applied Biosystems, Foster City, CA), and the results were analyzed using SDS Version 2.1 software (Austin Biodiversity Website Gallery).

**Nitro Blue Tetrazolium Slide Test**

Nitro blue tetrazolium (NBT) slide tests were performed as described previously (44). Briefly, a drop of whole blood was placed on each of two glass slides and incubated at 37 °C. Non-adherent cells were washed away with phosphate-buffered saline. Krebs-Ringer phosphate/glucose buffer with bovine serum albumin and NBT was added to each slide with or without phorbol 12-myristate 13-acetate (PMA; 20 μg/ml). Cells were incubated at 37 °C, washed with phosphate-buffered saline, fixed with methanol, and counterstained with 1% safranin. Cells were scored microscopically as the percentage of NBT-positive or NBT-negative of 200 cells counted.

**Statistical Analysis**

For reporter gene assays, groups of transfecants were initially analyzed by the technique of analysis of variance between groups. Conditions with reporter gene expression significantly different from the others were identified by calculating the F value and determining the p value for the null hypothesis (i.e. no difference between conditions). For individual pairs of transfectants, Student’s t test was used to determine the significance (p value) between individual data sets. Calculations were performed using SigmaPlot and SigmaStat software (Systat Software Inc., Richmond, CA).

**RESULTS**

**Expression of a Leukemia-associated Activating SHP2 PTP Mutant Blocks gp91PHOX and p67PHOX Expression in Differentiating Myeloid Cells**—In initial experiments, we determined the impact of expressing one of the previously described leukemia-associated activating SHP2 PTP mutants on gp91PHOX and p67PHOX expression in differentiating myeloid cells. For these experiments, we used an SHP2 mutant that has been described in myelodysplastic syndromes and AML in human subjects (E76K SHP2) (30). Stable transfectant pools of U937 myeloid cells were generated with vector expressing E76K SHP2 or empty control vector. Because transfection with this E76K SHP2 expression vector results in SHP2 overexpression relative to endogenous protein, stable transfectants were also generated with vector overexpressing wild-type SHP2 as a negative control. Three different transfecant pools were analyzed for each construct.
U937 is a leukemia cell line that can be induced to undergo monocyte differentiation upon treatment with IFN-γ (39). U937 differentiation is characterized by acquisition of mature phagocyte functions, including phagocytosis and respiratory burst oxidase activity. The latter reflects increased transcrip-
tion of the CYBB and NCF2 genes (45, 46). This cell line has relative overexpression of HoxA10 as one of the genetic lesions involved in the transformed phenotype (35). Therefore, these stable U937 transfectant pools were analyzed for gp91PHOX and p67PHOX expression during IFN-γ-induced differentiation. For these experiments, RNA isolated from transfectants with and without 48 h of IFN-γ treatment was analyzed by real-time PCR for gp91PHOX and p67PHOX message abundance. The results were normalized to 18 S RNA and actin abundance.

We found that overexpression of E76K SHP2 slightly decreased $g_{p91}^{\text{PHOX}}$ and $p_{67}^{\text{PHOX}}$ mRNAs in undifferentiated U937 cells in comparison with wild-type SHP2 or empty con-
trol vector. However, this decrease was not statistically significant for either $g_{p91}^{\text{PHOX}} (p = 0.06, F = 4.58, n = 3)$ or $p_{67}^{\text{PHOX}} (p = 0.072, F = 3.58, n = 3)$ (Fig. 1A). In contrast, in comparison with control vector-transfected cells, we found that E76K SHP2 overexpression significantly decreased the impact of IFN-γ differentiation on the abundance of $g_{p91}^{\text{PHOX}}$ mRNA (53.9 ± 6.2% less, $p = 0.0007, n = 3$) and $p_{67}^{\text{PHOX}}$ mRNA (71.7 ± 10.5% less, $p = 0.0004, n = 3$). There was no significant difference between wild-
type SHP2-overexpressing cells and transfectants with empty control vector in terms of $g_{p91}^{\text{PHOX}} (p = 0.97, n = 3)$ or $p_{67}^{\text{PHOX}} (p = 0.85, n = 3)$ mRNA abundance in differen-
tiated transfectants.

In control experiments, we dem-
strated an equivalent abundance of SHP2 mRNA in transfectants overexpressing either wild-type or E76K SHP2 (Fig. 1B). Consistent with this, we found equivalent pro-
tein overexpression of SHP2 in wild-type and E76K SHP2 transfec-
tants (Fig. 1C). Overexpression of activated or wild-type SHP2 did not impact endogenous HoxA10 protein abundance. Shown is a repre-
sentative blot from one of the three sets of stable transfectant pools.

Therefore, overexpression of con-
stitutively active (but not wild-type) SHP2 impairs expression of endog-
enous oxidase proteins.

However, U937 cells are a trans-
formed model of myeloid differenti-
ation. Therefore, we investigated the impact of overexpression of wild-type versus E76K SHP2 on $g_{p91}^{\text{PHOX}}$ and $p_{67}^{\text{PHOX}}$ expression in a non-transformed model, ex vivo differentiating murine myeloid progenitor cells. For these experiments, murine bone marrow myeloid progenitor cells were isolated and cultured with GM-CSF, stem cell factor, and IL-3. Cells were transduced with a retroviral vector expressing wild-type SHP2 or E76K SHP2 or with control vector. Transduced cells were selected in puromycin, and some cells were differentiated with M-CSF. Therefore, these experiments also allowed us to study differentiation in response to a different cytokine. Protein expression was analyzed by Western blotting of total cell lysate proteins (Fig. 2A). Shown is a representative blot from three independent experiments.

We found that expression of E76K SHP2 decreased differen-
tiation-induced expression of $g_{p91}^{\text{PHOX}}$ and $p_{67}^{\text{PHOX}}$ in these cells. In contrast, equivalent overexpression of wild-type SHP2
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FIGURE 2. Expression of a leukemia-associated mutant form of SHP2 inhibits gp91PHOX and p67PHOX expression and phagocyte oxidase activity in ex vivo differentiating murine myeloid progenitor cells. A, expression of E76K SHP2 inhibits induction of gp91PHOX and p67PHOX expression in murine myeloid progenitor cells undergoing ex vivo differentiation with M-CSF. Murine bone marrow myeloid progenitor cells were transduced with a retroviral vector to express wild-type or E76K SHP2 or with empty control vector. Progenitor cells were cultured in GM-CSF and IL-3 or ex vivo differentiated with M-CSF. Total cell lysates were separated by SDS-PAGE, and Western blots (WB) were serially probed with antibodies to gp91PHOX, p67PHOX, HoxA10, SHP2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as a loading control). In control vector cells or cells expressing wild-type SHP2, M-CSF differentiation induced gp91PHOX and p67PHOX protein expression. However, expression of these oxidase proteins was poorly induced during M-CSF differentiation of E76K SHP2-overexpressing cells. HoxA10 protein abundance was not influenced by SHP2 overexpression. MSCV, murine stem cell virus. B, expression of E76K SHP2 inhibits the respiratory burst activity of ex vivo M-CSF-differentiated murine myeloid progenitor cells. An aliquot of the transduced M-CSF-differentiated murine myeloid cells was also used to assay respiratory burst competence. Respiratory burst oxidase activation by PMA was determined by reduction of NBT. Cells not treated with PMA were used as a negative control for this assay. Cells expressing E76K SHP2 had impaired NBT reduction, consistent with decreased expression of rate-limiting oxidase components.

Expression of Leukemia-associated Activating SHP2 PTP Mutants Influences HoxA10 Repression of the CYBB and NCF2 Genes—On the basis of these results, we next investigated whether the effect of E76K SHP2 on gp91PHOX and p67PHOX expression is due to an impact on HoxA10 repression of CYBB and NCF2 transcription. In previous studies, we identified homologous negative cis-elements in the CYBB and NCF2 genes; HoxA10 represses these cis-elements only in undifferentiated myeloid cells (20, 21). Therefore, we determined the impact of E76K SHP2 expression on HoxA10 repression activity with and without IFN-γ treatment of the transfectants. For these experiments, we used artificial promoter constructs with multiple copies of the cis-element from the CYBB or NCF2 gene linked to a minimal promoter and a reporter (cybbTATACAT or ncf2TATACAT, respectively) as described (20, 21). U937 cells were transfected with these vectors or empty control vector (pTATACAT), with vector overexpressing HoxA10 or control vector, and with vector overexpressing wild-type or E76K SHP2 or control vector.

Because we were interested in the impact of E76K SHP2 on overexpressed HoxA10, we performed initial experiments to titrate the expression vector so that E76K SHP2 overexpression alone did not repress these cis-elements. Therefore, the reporter activity of the CYBB cis-element-containing construct was not significantly different in transfectants with wild-type SHP2, E76K SHP2, or empty control vector (p = 0.6, F = 0.6, n = 6) (Fig. 3A). Similarly, there was no difference in the reporter activity of the NCF2 cis-element-containing reporter construct in transfectants with wild-type SHP2, E76K SHP2, or empty control vector (p = 0.1, F = 3.3, n = 6) (Fig. 3B).

We next confirmed our previous results that overexpressed HoxA10 represses the negative CYBB and NCF2 cis-elements only in undifferentiated transfectants. We found that HoxA10 overexpression significantly repressed reporter expression via the CYBB cis-element in comparison with empty control vector in undifferentiated transfectants (39.1 ± 2.9% decrease, p = 0.001, n = 6) (Fig. 3A). Similarly, HoxA10 overexpression significantly repressed reporter expression via the negative NCF2 cis-element in undifferentiated U937 transfectants (30.5 ± 2.8% decrease, p = 0.0001, n = 6) (Fig. 3B). In contrast, overexpressed HoxA10 did not significantly repress reporter expression from the construct with either the CYBB or NCF2 cis-element in IFN-γ differentiated transfectants in comparison with empty control vector transfectants (p = 0.24, n = 6 versus p = 0.9, n = 6).

Therefore, we determined whether co-overexpression of wild-type or E76K SHP2 influences HoxA10-induced repression of either of these negative cis-elements. We found that repression of reporter expression from the CYBB (Fig. 3A) or NCF2 (Fig. 3B) cis-element-containing constructs in transfectants co-overexpressing wild-type SHP2 and HoxA10 was not significantly different from that in transfectants overexpressing...
FIGURE 3. Expression of E76K SHP2 increases HoxA10-induced repression of homologous cis-elements in the CYBB and NCF2 genes. A, E76K SHP2 expression increases HoxA10-induced repression of the negative cis-element in the CYBB promoter. U937 cells were transfected with a minimal promoter-reporter vector with four copies of the negative cis-element (cybbTATACAT) or reporter vector with a minimal promoter-reporter vector with four copies of the negative cis-element (cybbTATACAT) or with empty control vector (pTATACAT). Cells were cotransfected with vectors expressing various combinations of HoxA10, wild-type SHP2, and E76K SHP2 and with empty vector. Reporter gene assays were performed with and without IFN-γ (IFNg) differentiation. HoxA10 overexpression repressed this construct in untreated transfectants, but repression was not significantly different from untreated transfectants. Co-overexpression of HoxA10 and wild-type SHP2 significantly increased repression of the CYBB cis-element in comparison with HoxA10 expression alone. In contrast, only co-overexpression of HoxA10 and E76K SHP2 significantly repressed the cybbTATACAT construct in IFN-γ-treated transfectants. CAT, chloramphenicol acetyltransferase. B, E76K SHP2 expression increases HoxA10-induced repression of the negative cis-element in the NCF2 promoter. U937 cells were transfected with a minimal promoter-reporter vector with four copies of the negative cis-element from the NCF2 promoter (ncf2TATACAT) or with empty control vector (pTATACAT). Cells were cotransfected with vectors expressing various combinations of HoxA10, wild-type SHP2, and E76K SHP2 and with empty control vector. Reporter gene assays were performed with and without IFN-γ differentiation. HoxA10 overexpression repressed this construct in untreated transfectants, but repression was not significantly different from untreated transfectants. Co-overexpression of HoxA10 and wild-type SHP2 significantly increased repression of the NCF2 cis-element in comparison with HoxA10 overexpression alone. In contrast, only co-overexpression of HoxA10 and E76K SHP2 significantly repressed the ncf2TATACAT cis-element in the NCF2 promoter. U937 cells were transfected with a minimal promoter-reporter vector with four copies of the negative cis-element from the CYBB promoter (cybbTATACAT) or with empty control vector (pTATACAT). Cells were cotransfected with vectors expressing various combinations of HoxA10, wild-type SHP2, and E76K SHP2 and with empty control vector. Reporter gene assays were performed with and without IFN-γ differentiation. Similar to E76K SHP2, co-overexpression of HoxA10 and D61Y SHP2 significantly repressed reporter activity from this construct in comparison with HoxA10 alone. The effect of HoxA10 and D61Y SHP2 on cybbTATACAT reporter activity with and without IFN-γ differentiation was not significantly different from the effect of HoxA10 and E76K SHP2.
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activity. For these experiments, we used a mutant (D61Y SHP2) that induces a similar conformational change in SHP2, leading to constitutive activation (33). U937 cells were transfected with the CYBB cis-element-containing reporter construct or empty control vector, with vector overexpressing HoxA10 or a control vector, and with a vector expressing E76K SHP2 or D61Y SHP2 or control vector. Reporter activity was assayed with and without IFN-γ differentiation. In these experiments, we found that repression of the CYBB cis-element by HoxA10 and D61Y SHP2 was not significantly different from that by HoxA10 and E76K SHP2 without (p = 0.96, n = 3) or with (p = 0.79, n = 3) IFN-γ differentiation of the transfectants (Fig. 3C). These results suggest that activating mutants that induce similar conformational changes may have a similar impact on HoxA10 transcriprional repression activity.

*Endogenous SHP2 PTP Activity Influences HoxA10 Repression of the CYBB and NCF2 Genes in Undifferentiated Myeloid Cells*—We were interested in determining whether HoxA10 is a substrate for wild-type SHP2. Alternatively, HoxA10 might be a substrate for only the leukemia-associated constitutively active form of SHP2. To investigate these two possibilities, we used a previously described phosphatase-inactive dominant-negative form of SHP2 (C463S SHP2) (48). In these experiments, U937 cells were cotransfected with the same negative CYBB or NCF2 cis-element-containing reporter vectors as described for the above experiments (cybbTATACAT, ncf2TATACAT, or pTATACAT), with vector overexpressing HoxA10 or empty control vector, and with vector expressing C463S SHP2 or empty control vector.

We found that overexpression of C463S SHP2 significantly increased reporter expression via the CYBB cis-element in comparison with empty control vector transfectants (39.8 ± 1.6% increase in reporter activity, p = 0.001, n = 4) (Fig. 4A). In addition, we found that the reporter activity of the CYBB cis-element-containing construct was significantly greater in transfectants co-overexpressing HoxA10 and C463S SHP2 than in transfectants expressing HoxA10 alone (121.0 ± 6.1% increase, p < 0.0001, n = 4). This relative activation in comparison with the pTATACAT vector is due to the presence of a positive cis-element overlapping the HoxA10-binding negative cis-element in the cybbTATACAT construct (49).

Similarly, we found that C463S SHP2 expression ablated the repression activity of the NCF2 cis-element in ncf2TATACAT in comparison with control vector (49.9 ± 8.6% increase in reporter activity, p < 0.001, n = 4) (Fig. 4B). Unlike cybbTATACAT, this construct has net repression activity because there is no adjacent overlapping positive cis-element in the NCF2 promoter (21). Additionally, reporter expression from this NCF2 cis-element-containing construct was significantly greater in transfectants coexpressing C463S SHP2 and HoxA10 than in transfectants overexpressing HoxA10 alone (89.4 ± 10.2% increase, p < 0.001, n = 4).

Therefore, for both the CYBB and NCF2 cis-elements, co-overexpression of dominant-negative SHP2 and HoxA10 reversed the repression activity of HoxA10 overexpression alone. In control experiments, reporter expression from the pTATACAT control vector was not altered by overexpression of any of these proteins with or without IFN-γ differentiation.

Expression of a dominant-negative form of SHP2 decreases HoxA10-induced repression of negative cis-elements in the CYBB and NCF2 promoters. A, expression of a dominant-negative form of SHP2 (C463S SHP2) decreases HoxA10-induced repression of the negative cis-element in the CYBB promoter. U937 cells were transfected with a minimal promoter-reporter vector with four copies of the negative cis-element from the CYBB promoter (cybbTATACAT) or with empty control vector (pTATACAT). Cells were cotransfected with vectors overexpressing various combinations of HoxA10 and C463S SHP2 and with empty control vector. Reporter expression from the CYBB cis-element-containing construct was increased significantly in transfectants overexpressing C463S SHP2 in comparison with control vector transfectants. Additionally, co-overexpression of C463S SHP2 reversed repression of the CYBB cis-element by overexpressed HoxA10. Reporter activity in cybbTATACAT transfectants co-overexpressing HoxA10 and C463S SHP2 was not significantly different from that in transfectants overexpressing C463S SHP2 alone. B, expression of a dominant-negative form of SHP2 (C463S SHP2) decreases HoxA10-induced repression of the negative cis-element in the NCF2 promoter. U937 cells were transfected with a minimal promoter-reporter vector with four copies of the negative cis-element from the NCF2 promoter (ncf2TATACAT) or with empty control vector (pTATACAT). Cells were cotransfected with vectors overexpressing various combinations of HoxA10 and C463S SHP2 and with empty control vector. Reporter expression from the NCF2 cis-element-containing construct was significantly increased in transfectants overexpressing C463S SHP2 in comparison with control vector transfectants. Additionally, co-overexpression of C463S SHP2 reversed repression of the NCF2 cis-element by overexpressed HoxA10. Reporter activity in ncf2TATACAT transfectants co-overexpressing HoxA10 and C463S SHP2 was not significantly different from that in transfectants overexpressing C463S SHP2 alone.
cross-links were generated in vivo before and after IFN-γ differentiation. Cell lysates were immunoprecipitated with HoxA10 antiserum or control preimmune serum, and coprecipitated chromatin was PCR-amplified with primers flanking the HoxA10-binding cis-element in the CYBB or NCF2 promoter. Amplified DNA was identified on acrylamide gels with input (non-precipitated) chromatin as a positive control. Consistent with expectations, we observed in vivo HoxA10 binding to both the CYBB (Fig. 5A) and NCF2 (Fig. 5B) cis-elements in undifferentiated control U937 transfectants. Shown are representative results from two independent experiments. The interaction of HoxA10 with these promoters was slightly increased in stable transfectants with E76K SHP2.

In assays with IFN-γ-treated cells, HoxA10 did not interact with the CYBB (Fig. 5A) or NCF2 (Fig. 5B) cis-element in transfectants with empty control vector or the wild-type SHP2 expression vector. In contrast, we found that IFN-γ treatment did not significantly influence in vivo HoxA10 binding to either the CYBB (Fig. 5A) or NCF2 (Fig. 5B) cis-element in stable U937 transfectants expressing E76K SHP2.

On the basis of these results, we also tested the impact of E76K SHP2 expression on HoxA10 binding to the CYBB and NCF2 cis-elements by EMSAs. For these experiments, nuclear proteins were isolated from stable U937 transfectants expressing E76K SHP2 or empty control vector before and after 48 h of IFN-γ treatment. These proteins were used in EMSAs with radiolabeled probes representing the negative cis-element from the CYBB or NCF2 gene. In assays with proteins from undifferentiated E76K SHP2-overexpressing control transfectants, this probe bound a low mobility HoxA10-containing protein complex (upper arrow). Binding of this complex decreased in assays with nuclear proteins from IFN-γ-treated control transfectants, but not from E76K SHP2-overexpressing transfectants. Binding of a higher mobility complex increased in assays with nuclear proteins from IFN-γ-treated control transfectants (middle arrow). The asterisk indicates binding of the classical CCAAT factor CP1 to the CYBB promoter, and the lower arrow indicates the free probe. ISRE, interferon-stimulated response element. D, E76K SHP2 expression increases in vivo binding of a HoxA10-containing protein complex to the negative CYBB cis-element in IFN-γ-treated U937 cells. Nuclear proteins were isolated from untreated or IFN-γ-differentiated U937 cells stably transfected with vector overexpressing E76K SHP2 or with control vector. These proteins were used in EMSAs with a radiolabeled probe representing the negative cis-element from the CYBB gene. In assays with proteins from undifferentiated E76K SHP2-overexpressing or control transfectants, this probe bound a low mobility HoxA10-containing protein complex (upper arrow). Binding of this complex decreased in assays with nuclear proteins from IFN-γ-treated control transfectants, but not from E76K SHP2-overexpressing transfectants. Binding of a higher mobility complex increased in assays with nuclear proteins from IFN-γ-treated control transfectants (middle arrow). The asterisk indicates binding of the classical CCAAT factor CP1 to the CYBB promoter, and the lower arrow indicates the free probe. E, overexpression of E76K SHP2 increases in vivo HoxA10 binding to the CYBB and NCF2 cis-elements in assays with nuclear proteins from IFN-γ-treated transfectants. EMSAs were performed with the CYBB or NCF2 cis-element probe and nuclear proteins from IFN-γ-treated stable U937 transfectants overexpressing E76K SHP2. Binding assays were preincubated with HoxA10 antiserum or preimmune serum control. Binding of the low mobility complex was disrupted in binding assays with HoxA10-specific antibody.

We observed binding of a low mobility complex in EMSAs with the CYBB cis-element probe and nuclear proteins from untreated control U937 transfectants, but not from IFN-γ-treated transfectants (Fig. 5C). We previously demonstrated that this is a specific complex that includes HoxA10, Pbx1, and the negative

binding to the CYBB and NCF2 promoters by in vitro and in vivo assays.

We first tested in vivo HoxA10-DNA binding in cells overexpressing wild-type or E76K SHP2 using the stable U937 transfectants as described above (Fig. 1, B and C). Protein-DNA...
HDAC2 (20, 24, 28). In IFN-γ-differentiated control transfectants, decreased binding of the low mobility complex was accompanied by increased binding of a higher mobility complex. This latter complex represents protein binding to the overlapping positive cis-element in this probe (49). In contrast, binding of the HoxA10-containing protein complex did not decrease in EMSAs with nuclear proteins from IFN-γ-treated U937 transfectants overexpressing E76K SHP2 (Fig. 5C). In EMSAs with the NCF2 cis-element probe and nuclear proteins from control U937 transfectants, IFN-γ treatment similarly decreased binding of the HoxA10-containing complex to this probe (Fig. 5D). Binding of this complex to the NCF2 cis-element probe also persisted in EMSAs with nuclear proteins from IFN-γ-treated stable U937 transfectants expressing E76K SHP2 (Fig. 5D).

These results suggest that E76K SHP2 expression blocks the IFN-γ-induced decrease in HoxA10 binding to the CYBB and NCF2 cis-elements. Alternatively, expression of E76K SHP2 might induce binding of different proteins to these cis-elements. Therefore, we verified that HoxA10 is a component of the complex that binds the CYBB or NCF2 cis-element in EMSAs with nuclear proteins from IFN-γ-treated stable U937 transfectants overexpressing E76K SHP2. To do this, nuclear proteins were preincubated with HoxA10 antiserum or preimmune serum prior to incubation with the CYBB or NCF2 probe. We found that incubation with HoxA10 antiserum (but not preimmune serum) disrupted binding of this low mobility protein complex to both the CYBB and NCF2 cis-element probes (Fig. 5E).

Expression of a Leukemia-associated Activating SHP2 PTP Mutant Influences HoxA10 Tyrosine Phosphorylation—These results suggest that wild-type SHP2 dephosphorylates HoxA10 in undifferentiated myeloid cells, but that E76K SHP2 dephosphorylates HoxA10 in undifferentiated and differentiating myeloid cells. To investigate this, we determined the phosphorylation state of HoxA10 in stable U937 transfectants overexpressing these proteins or transfected with empty control vector. For these experiments, nuclear proteins were isolated from stable U937 transfectants before and after IFN-γ differentiation. These proteins were immunoprecipitated under denaturing conditions with HoxA10 antiserum or control preimmune serum. Immunoprecipitates were separated by SDS-PAGE, and Western blots were serially probed with anti-phosphotyrosine antibody followed by anti-HoxA10 antibody. IFN-γ treatment increased tyrosine phosphorylation of HoxA10 in assays with proteins from control or SHP2-overexpressing U937 transfectants. In contrast, HoxA10 tyrosine phosphorylation did not increase in assays with proteins from IFN-γ-treated U937 transfectants overexpressing E76K SHP2. Overexpression of either wild-type or E76K SHP2 significantly altered expression of endogenous HoxA10.

In contrast, HoxA10 tyrosine phosphorylation is inhibited by E76K SHP2 overexpression in IFN-γ-treated U937 cells. Nuclear proteins were isolated from stable U937 transfectants overexpressing wild-type or E76K SHP2 or were transfected with empty vector with and without IFN-γ differentiation. These proteins were immunoprecipitated (IP) under denaturing conditions with HoxA10 antiserum or control preimmune serum. Immunoprecipitates were separated by SDS-PAGE, and Western blots were serially probed with anti-phosphotyrosine antibody (α PY) followed by anti-HoxA10 antibody. IFN-γ treatment increased tyrosine phosphorylation (but not total) HoxA10 in assays with proteins from control or SHP2-overexpressing U937 transfectants. In contrast, HoxA10 tyrosine phosphorylation did not increase in assays with proteins from IFN-γ-treated U937 transfectants overexpressing E76K SHP2. Overexpression of either wild-type or E76K SHP2 significantly altered expression of endogenous HoxA10. Thus, HoxA10 tyrosine phosphorylation is inhibited by E76K SHP2 overexpression in ex vivo M-CSF-differentiated murine myeloid progenitor cells. Murine bone marrow myeloid progenitor cells were transduced with a retroviral vector expressing wild-type or E76K SHP2 or with empty control vector. Progenitor cells were cultured in GM-CSF and IL-3 or ex vivo differentiated with M-CSF. Lysate proteins were immunoprecipitated under denaturing conditions with anti-phosphotyrosine antibody or irrelevant control antibody. Immunoprecipitates were separated by SDS-PAGE, and Western blots were probed with anti-HoxA10 antibody. M-CSF differentiation increased HoxA10 tyrosine phosphorylation in control vector and wild-type SHP2-overexpressing cells. In contrast, HoxA10 was not tyrosine-phosphorylated in M-CSF-differentiated cells overexpressing E76K SHP2.

These results demonstrate HoxA10 tyrosine phosphorylation in response to M-CSF differentiation in cells transduced with the control or wild-type SHP2 expression vector (Fig. 6B). In contrast, we found no increase in HoxA10 tyrosine phosphorylation in M-CSF-differentiated myeloid progenitor cells overexpressing E76K SHP2.

HoxA10 Is a Substrate for SHP2—Although these results suggest that E76K SHP2 expression influences HoxA10 tyrosine phosphorylation in differentiating myeloid cells, they do not demonstrate that HoxA10 is a substrate for E76K SHP2. An alternative possibility would be that E76K SHP2 activates or
HoxA10 interacts with and is a substrate for SHP2. A, HoxA10 co-immunoprecipitates from U937 nuclear proteins with SHP1 and SHP2. Nuclear proteins were isolated from U937 cells and immunoprecipitated (IP) with anti-SHP2 antibody under non-denaturing conditions. U937 nuclear proteins were also immunoprecipitated with anti-SHP1 antibody as a positive control and with an irrelevant antibody as a negative control. Immunoprecipitates were separated by SDS-PAGE, and Western blots (WB) were serially probed with antibodies to SHP1, SHP2, and HoxA10. HoxA10 co-immunoprecipitated with anti-SHP1 antibody, consistent with our previous results (41). HoxA10 also coprecipitated with anti-SHP2 antibody, but not with irrelevant control antibody. B, in vitro translated HoxA10 copurifies with either SHP1 or SHP2 expressed as a GST fusion protein. 35S-Labeled HoxA10 was in vitro translated in reticulocyte lysate and used in affinity purification assays with SHP1 or SHP2 expressed as a GST fusion protein in E. coli. GST protein was used as a negative control in these experiments. In vitro HoxA10 was incubated with SHP1-GST, SHP2-GST, or GST control; the GST proteins were affinity-purified under non-denaturing conditions on glutathione-agarose; and the affinity-purified proteins were separated by SDS-PAGE. HoxA10 was identified by autoradiography of the acrylamide gel. HoxA10 coprecipitated with SHP1-GST and SHP2-GST, but not with control GST. C, in vitro translated HoxA10 is dephosphorylated by in vitro translated SHP2. 35S-Labeled HoxA10 was in vitro translated in reticulocyte lysate and incubated with either in vitro translated (IVT) E76K SHP2 (unlabeled) or control reticulocyte lysate. Reaction mixtures were immunoprecipitated under denaturing conditions with anti-phosphotyrosine antibody or control antibody. Immunoprecipitates were separated by SDS-PAGE, and HoxA10 was identified by autoradiography of the acrylamide gel. In vitro translated HoxA10 incubated with control lysate was tyrosine-phosphorylated, consistent with our previous results (28). In contrast, HoxA10 incubated with E76K SHP2 did not immunoprecipitate with anti-phosphotyrosine antibody. D, in vitro translated E76K SHP2 dephosphorylates HoxA10 immunoprecipitated from U937 nuclear proteins. Nuclear extract (NE) proteins from IFN-γ-differentiated U937 cells were immunoprecipitated under denaturing conditions with HoxA10 antiserum or control preimmune serum. Immunoprecipitates were collected, and renatured proteins were incubated with in vitro translated E76K SHP2 or C4635 SHP2. Immunoprecipitates were separated by SDS-PAGE, and Western blots were serially probed with antibodies to phosphotyrosine (α-P-Y) and HoxA10. In comparison with incubation with PTP-inactive SHP2, incubation with E76K SHP2 decreased tyrosine phosphorylation of HoxA10 isolated from differentiated U937 cells.

In initial experiments, we determined whether endogenous HoxA10 interacts with endogenous SHP2 in U937 myeloid cells. For these experiments, U937 nuclear proteins were immunoprecipitated under non-denaturing conditions with anti-SHP2 antibody. Nuclear proteins were also immunoprecipitated with anti-SHP1 antibody as a positive control and with an irrelevant antibody as a negative control. Immunoprecipitates were separated by SDS-PAGE, and Western blots were serially probed with antibodies to HoxA10, SHP1, and SHP2. A representative blot from one of three independent experiments is shown. We found that HoxA10 specifically co-immunoprecipitated with either SHP2 or SHP1.

However, it is possible that HoxA10 and SHP2 do not interact directly, but via a third unidentified protein. To investigate this, we studied the interaction between in vitro translated HoxA10 and SHP2 expressed in E. coli as a fusion protein with GST (SHP2-GST) by pull-down assay. For these experiments, SHP1-GST was used as a positive control, and GST protein was used as a negative control for HoxA10 coprecipitation. SHP2-GST, SHP1-GST, or control GST was incubated with in vitro translated 35S-labeled HoxA10, and incubation mixtures were affinity-purified on glutathione-agarose. Precipitates were separated by SDS-PAGE, and proteins were identified by Coomassie Blue staining and autoradiography. This experiment was repeated more than three times, and representative results are shown. We found that HoxA10 coprecipitated at least as efficiently with SHP2-GST as with SHP1-GST (Fig. 6B). In contrast, HoxA10 did not coprecipitate with GST protein alone.

Therefore, we next investigated the ability of recombinant SHP2 to dephosphorylate recombinant HoxA10. For these experiments, HoxA10 and E76K SHP2 were expressed as in vitro translated proteins. 35S-Labeled HoxA10 was incubated with unlabeled E76K SHP2 or control lysate. Proteins were immunoprecipitated under denaturing conditions with anti-phosphotyrosine antibody or irrelevant control antibody. Immunoprecipitates were separated by SDS-PAGE, and tyrosine-phosphorylated proteins were identified by autoradiography. This experiment was repeated in duplicate, and a representative autoradiograph is shown. We found that preincubation of HoxA10 with control lysate did not decrease tyrosine phosphorylation (Fig. 7C), consistent with our previous results (28). In contrast, HoxA10 incubated with in vitro translated E76K SHP2 did not immunoprecipitate with anti-phosphotyrosine antibody. These results suggest that HoxA10 is a substrate for SHP2.

However, the HoxA10 tyrosine residues that are phosphorylated in vitro in reticulocyte lysate may not be the same as the residues phosphorylated in IFN-γ-treated U937 cells. Therefore, we determined the ability of in vitro translated E76K SHP2 to dephosphorylate HoxA10 isolated from U937 cells. For these experiments, HoxA10 was immunoprecipitated

inhibits a third protein that dephosphorylates HoxA10.

In initial experiments, we determined whether endogenous HoxA10 interacts with endogenous SHP2 in U937 myeloid cells. For these experiments, U937 nuclear proteins were immunoprecipitated under non-denaturing conditions with anti-SHP2 antibody. Nuclear proteins were also immunoprecipitated with anti-SHP1 antibody as a positive control and with an irrelevant antibody as a negative control. Immunoprecipitates were separated by SDS-PAGE, and Western blots were serially probed with antibodies to HoxA10, SHP1, and SHP2. A representative blot from one of three independent experiments is shown. We found that HoxA10 specifically co-immunoprecipitated with either SHP2 or SHP1.
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from IFN-γ-differentiated U937 nuclear proteins under denaturing conditions with anti-HoxA10 antibody or control preimmune serum. Immunoprecipitates were collected, renatured, and incubated with in vitro translated SHP2 (non-[35S]methionine-labeled). For these experiments, E76K SHP2 was compared with phosphatase-inactive C463S SHP2, which was used as a negative control. Immunoprecipitates from these incubation reactions were washed extensively and separated by SDS-PAGE. Western blots were serially probed with anti-phosphotyrosine and anti-HoxA10 antibodies (Fig. 7D). Incubation with E76K SHP2 decreased the abundance of tyrosine-phosphorylated (but not total) HoxA10 in comparison with incubation with PTP-inactive SHP2.

DISCUSSION

Our study has demonstrated that both SHP1 and SHP2 are involved in maintaining HoxA10 in a non-tyrosine-phosphorylated state in undifferentiated myeloid cells. This effect on HoxA10 tyrosine phosphorylation is abolished by unknown mechanisms during myelopoiesis. This study has also shown that HoxA10 is a substrate for a leukemia-associated constitutively active mutant form of SHP2 in both undifferentiated and differentiating myeloid cells. Expression of activated SHP2 therefore results in persistent repression of CYBB and NCF2 transcription in differentiating myeloid cells. Because HoxA10 overexpression and SHP2 activation are both leukemia-associated mutations, our results identify a novel mechanism by which these mutations might cooperate and result in functional differentiation block in leukemic myelopoiesis.

Transcription of the CYBB and NCF2 genes occurs concurrently during myelopoiesis and in response to inflammatory mediators in mature phagocytes (50). Consistent with this, transcription is regulated by homologous CYBB and NCF2 cis-elements that interact with a common set of transcription factors. In this study, we investigated the homologous negative cis-elements that are repressed by HoxA10 (20, 21, 24). Additionally, homologous positive cis-elements in the proximal promoters of these genes are activated by a multiprotein complex that includes PU.1, interferon regulatory factor-1, interferon consensus sequence-binding protein (ICSBP), and the cAMP-responsive element-binding protein-binding protein (13, 45, 46, 51).

Despite the presence of these homologous cis-elements, there are differences in regulation of the CYBB and NCF2 promoters. For example, the HoxA10-binding repressor element in the CYBB gene overlaps a positive cis-element with an interferon-stimulated response element consensus sequence (49). Therefore, HoxA10 represses CYBB transcription by two mechanisms: endogenous repression due to interaction with HDAC2 and competition with transcriptional activators for occupancy of the overlapping interferon-stimulated response element (20). In contrast, the repressor element in the NCF2 gene does not overlap a positive cis-element. Therefore, HoxA10 represses NCF2 transcription by only one mechanism: HDAC2 recruitment (21). These differences are reflected in the activities of these cis-elements in the functional assays in this study.

HoxA10 is tyrosine-phosphorylated in response to signaling events triggered by hematopoietic cytokines. Therefore, repression of the CYBB and NCF2 genes is reversible and regulated by cytokine-stimulated signaling pathways. The balance could be shifted toward decreased gpg91[ΔHOX] and p69[ΔHOX] expression by phosphatase activity and toward increased expression by kinase activity. In this study, we found that expression of leukemia-associated activating SHP2 mutants impairs the normal cytokine-stimulated regulation of this balance.

Although SHP2 PTP activity is known to be involved in leukemia, few substrates that mediate these effects have been identified. One such substrate is ICSBP (37). Expression of constitutively active SHP2 dephosphorylates ICSBP in differentiating myeloid cells. This impairs ICSBP activation of the gene encoding neurofibromin 1 and results in cytokine hypersensitivity (37, 42). In this study, we identified HoxA10 as another SHP2 substrate that may contribute to the transformed phenotype in malignant myeloid cells. The results of this study suggest that expression of activating mutations of SHP2 would result in repression of myeloid-specific gene transcription by overexpressed HoxA10. This could either impair respiratory burst activity in these cells or contribute to differentiation block and therefore AML.

We found that the impact of SHP1 and SHP2 on HoxA10 tyrosine phosphorylation in differentiated myeloid cells is minimal. Because this is not the case for the activating SHP2 mutants, these results suggest that events downstream from cytokine stimulation influence the conformational changes involved in unmasking the PTP domain in the wild-type protein. The mechanisms that regulate SHP1 and SHP2 PTP activities are not known. Although these PTPs are tyrosine-phosphorylated in response to various cytokines, the functional consequences of this phosphorylation are not clear (51). This mechanism will be of interest.

Therefore, this study has identified HoxA10 overexpression and constitutive SHP2 activation as potential cooperating mutations leading to differentiation block in malignant myeloid cells. We have identified a novel mechanism by which mutation in a signaling pathway increases the impact of aberrant Hox protein abundance. Identification of such collaborating mutations will be of interest in defining potential pathways for therapeutic interventions in myeloid malignancies.

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SHP2 PTP Inhibits CYBB and NCF2 Transcription