Identification of a HoxA10 Activation Domain Necessary for Transcription of the Gene Encoding β3 Integrin during Myeloid Differentiation*

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Transcription of the ITGB3 gene, which encodes β3 integrin, increases during myeloid differentiation. αvβ3 integrin mediates adhesion to fibronectin or vitronectin and regulates various aspects of the inflammatory response in mature phagocytes. In these studies, we found that the homeodomain transcription factor HoxA10 interacted with a specific ITGB3 cis element and activated transcription of this gene during myeloid differentiation. We also found that increased fibronectin adhesion in differentiating myeloid cells was dependent upon this HoxA10-induced increase in β3 integrin expression. We determined that activation of ITGB3 transcription required a HoxA10 domain that was not identical to the “hexapeptide” that mediates interaction of Hox and Pbx proteins. This activation domain was also not identical to a previously identified HoxA10 repression domain that mediates interaction with transcriptional co-repressors. Instead, this HoxA10 activation domain had homology to “PQ” protein-protein interaction domains that have been described previously in other transcription factors. Consistent with this, we found that the HoxA10 PQ-like domain recruited the CREB-binding protein (CBP) to the ITGB3 promoter. This was associated with an increase in local histone acetylation in vivo. In immature myeloid cells, we previously determined that HoxA10 repressed transcription of the CYBB and NCF2 genes, which encode the phagocyte oxidase proteins gp91PHOX and p67PHOX, respectively. Therefore, our studies indicated that HoxA10 either activates or represses gene transcription at various points during myelopoiesis. Our studies also suggested that HoxA10 is a bifunctional protein that is involved in dynamic regulation of multiple aspects of phagocyte phenotype and function.

β3 integrin, which is encoded by the ITGB3 gene, is expressed in a variety of cell types. ITGB3 transcription occurs in hematopoietic stem cells (HSC)² and in various blood cell lineages (1). In cells of the myeloid lineage, β3 integrin participates in αvβ3 heterodimers that mediate cell adhesion to fibronectin and vitronectin (2, 3). Fibronectin is a component of the bone marrow extracellular matrix, and fibronectin binding promotes survival of HSC and differentiating myeloid progenitor cells (4, 5). Expression of β3 integrin increases during differentiation of neutrophils and monocytes (1). In mature monocytes, αvβ3 mediates phagocytosis of apoptotic neutrophils, thereby contributing to down-regulation of the inflammatory response (6, 7). In neutrophils, αvβ3 provides a “braking” mechanism during transmigration from the vascular space and is also involved in activation of the phagocyte-specific respiratory burst oxidase (8, 9). In addition to participation in the innate immune response, αvβ3-ligand binding activates Syk protein-tyrosine kinase and may thereby be involved in generation of cell survival signals in phagocytic cells (8).

These results suggest that understanding the mechanisms that regulate αvβ3 expression would be relevant to understanding phagocytic cell function and survival. However, regulation of ITGB3 transcription during myelopoiesis or in mature myeloid cells has not been studied. Indeed, the only previous studies of the ITGB3 promoter were performed in epithelial carcinoma cells (10). These studies identified a region of the ITGB3 promoter that interacts in vitro with the homeodomain transcription factor HoxA10. In these studies, overexpressed HoxA10 activated an artificial promoter construct with this region of the ITGB3 promoter in transfection experiments with a breast cancer cell line (10). Because HoxA10 is expressed in differentiating myeloid cells, the first hypothesis of our current studies was that HoxA10 regulates ITGB3 transcription during myelopoiesis.

The HOX genes are arranged in four paralog groups (A–D) on four different chromosomes in mouse and man. During embryogenesis, HOX gene transcription occurs 3’ to 5’ through each paralog group with the more 3’ genes expressed in cephalad and the more 5’ genes in caudal organs (11). HOX genes are also sequentially expressed during definitive hematopoiesis with the 3’-most genes being expressed in HSCs and 5’-most genes in more differentiated hematopoietic cells (12). Consistent with this, ABD HOXA genes (HoxA7–11) are maximally expressed in committed myeloid progenitors. Abnormal stimulating factor; M-CSF, macrophage-CSF; EMSA, electrophoretic mobility shift assay; aa, amino acid; IL, interleukin.

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2 The abbreviations used are: HSC, hematopoietic stem cells; IFN, interferon; shRNA, short hairpin RNA; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage-CSF; EMSA, electrophoretic mobility shift assay; aa, amino acid; IL, interleukin.
expression of various Hox proteins is associated with acute myeloid leukemia, implying that tight regulation of the Abd HoxA proteins is important for normal myelopoiesis (13). Additionally, our previous studies indicated that Abd HoxA proteins are involved in regulating transcription of genes involved in phagocyte-specific functional activities (14, 15).

Therefore, the second hypothesis of these studies was that dynamic regulation of ITGB3 transcription by HoxA10 contributes to phagocyte function and survival during myelopoiesis. In our previous studies, we found that HoxA10 dynamically regulates transcription of the genes encoding the phagocyte oxidase proteins gp91PHOX and p67PHOX (the CYBB and NCF2 genes, respectively) (14, 15). These genes are expressed in myeloid cells after the promyelocyte stage of differentiation and are actively transcribed until cell death (16, 17). We determined that HoxA10 represses CYBB and NCF2 transcription in undifferentiated myeloid cells. In these cells, HoxA10 binds to homologous CYBB and NCF2 cis elements as a heterodimer with the homeodomain protein Pbx1 (14, 15).

Hox and Pbx proteins are frequent DNA-binding partners, and transcriptional regulation by some such heterodimers is mediated by Pbx interaction with transcriptional co-activators or co-repressors (18). However, we found that HoxA10 repression of CYBB and NCF2 transcription was Pbx-independent and involved direct interaction of HoxA10 with the co-repressor histone deacetylase 2 (HDAC2) (15, 19). This interaction required a unique HoxA10 domain, not conserved in other Abd HoxA proteins (19). As differentiation proceeds, HoxA10 binding to the CYBB and NCF2 genes decreases, relieving repression (14, 15). Because gp91PHOX and p67PHOX are the rate-limiting phagocyte oxidase components, this results in differentiation stage-specific expression of respiratory burst competence in mature phagocytes. If HoxA10 also regulates ITGB3 transcription in a differentiation stage-specific manner, this would further suggest that HoxA10 is an important regulator of phagocyte function.

Target genes that are activated by HoxA10 in myeloid cells have not been previously identified. However, based on previous studies, HoxA10 would be anticipated to activate ITGB3 transcription during myelopoiesis. Therefore, the first goal of these studies was to determine whether HoxA10 increases β3 integrin expression and ITGB3-transcription in differentiating myeloid cells. The second goal of these studies was to determine the functional relevance of Hox10 regulation of β3 integrin expression for differentiation-induced phagocytic adhesion. The third goal of these studies was to determine whether HoxA10 activation of ITGB3 transcription requires a Pbx partner. If transcriptional activation is not Pbx-dependent, ITGB3 transcription will be used as a model to identify a HoxA10 activation domain. Therefore, these studies address mechanisms regulating phagocyte function and also fundamental mechanisms of HoxA10 transcription factor activity.

MATERIALS AND METHODS

Plasmids and PCR Mutagenesis

Artificial Promoter Constructs—Artificial promoter/reporter constructs were generated as described previously (14, 15), in the minimal promoter/reporter vector, p-TATACAT (20) (obtained from Dr. A. Kraft, Hollings Cancer Center at the Medical University of South Carolina, Charleston). Constructs were generated with four copies (in the forward direction) of the −1973 to −1933 bp sequence from the ITGB3 promoter (p-B3ITATACAT) (10, 21).

cDNA Sequences and Mutagenesis—The cDNA for human HoxA10 was obtained from C. Largman (University of California, San Francisco) (22). This cDNA sequence represents the major transcript in mammalian hematopoietic cells, encoding a 393-amino acid, 55-kDa protein (22). Wild type HoxA10 cDNA sequence was subcloned into the pSRe vector for expression in mammalian cells (23). Wild type HoxA10 was also subcloned into the pM2 vector, for expression in mammalian cells as a fusion protein with the DNA-binding domain of the yeast Gal4 transcription factor GAL4, as described previously (14).

HoxA10 5′ truncation mutant cDNAs were generated by PCR, using primers that incorporate the genuine HoxA10 Kozak consensus sequence and an ATG. Truncation mutants were generated that included HoxA10 amino acids (aa) 1–219, 1–179, 1–112, 102–146, 124–146, and 145–219. These sequences were subcloned into the PM2 vector for expression as a fusion protein with the DNA-binding domain of the yeast Gal4 transcription factor. HoxA10 truncations were also generated representing amino acids 219–403, 146–403, and 60–403. These sequences were subcloned into the pSRa vector for expression in mammalian cells. HoxA10 cDNAs with mutation of the Pbx1 interaction domain were generated by PCR-based site-directed mutagenesis, by a previously described technique (19). The wild type HoxA10 was mutated to change amino acid 312 from asparagine to alanine and amino acid 313 from tryptophan to threonine (N312A/W323T HoxA10). This mutant cDNA was subcloned into a pSRa vector for expression in U937 transfection experiments. All mutant cDNA sequences generated by PCR were completely sequenced on both strands to verify that no unintended mutations had been introduced.

Plasmids for shRNA Expression—Plasmids were generated to express shRNA to human β3 integrin or HoxA10 using the pLKO.1 vector (kindly provided by Dr. K. Rundell, Northwestern University, Chicago). Oligonucleotide sequences for β3 integrin-specific shRNA, HoxA10-specific shRNA, or scrambled control shRNAs were designed with the assistance of the software on the Promega web site (Promega, Madison, WI).

Oligonucleotides

Oligonucleotides were synthesized by the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University. Double-stranded, synthetic oligonucleotides were generated representing the −1973 to −1933 bp sequence of the ITGB3 promoter (5′-GGGGGGGTTTATATAGTT-TATTTTTGTACCTTTACTAC-3′) or the −94 to −134 bp sequence of the CYBB promoter, 5′-TTTACTTGAATATATACTGTTAC-3′. In these oligonucleotides, the HoxA10 core is in boldface type; the Pbx core is in italic type, and the ccaat boxes are underlined. In some experiments, an irrelevant control sequence from the NFI promoter was also used.
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Myeloid Cell Line Culture

The human myelomonocytic cell line U937 (24) was obtained from Andrew Kraft (Hollings Cancer Center at the Medical University of South Carolina, Charleston). Cells were maintained and differentiated as described (14, 15, 19). U937 cells were treated with 500 units per ml of human recombinant IFN-γ for 24 or 48 h, as indicated (Hoffmann-La Roche).

Murine Bone Marrow Cell Culture

Bone marrow was harvested from HoxA10+/− mice or wild type litter mates, and myeloid progenitor cells were cultured in GM-CSF (20 ng/ml), IL3 (10 ng/ml), and stem cell factor (10 ng/ml) for 48 h. These cells were differentiated ex vivo in M-CSF (20 ng/ml) for 72 h. Animal experiments were performed according to a protocol approved by the Northwestern University Animal Care and Use Committee.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extract proteins were prepared by the method of Dignam et al. (25), with protease inhibitors as described previously (14). Oligonucleotides probes were prepared; EMSA and antibody supershift assays were performed, as described (14, 15). Antiserum to HoxA10 (not cross-reactive with other Hox proteins) was obtained from Covance Research Products (Richmond, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Pbx1 and Pbx2 (noncross-reactive with each other or other Pbx proteins) was obtained from Covance Research Products (Richmond, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA). Antiserum to HoxA10 (not cross-reactive with other Hox proteins) was obtained with 60-403-aa HoxA10/pSR, 15. Antibodies to tubulin, HoxA10, Syk, and phospho-Syk were obtained from Santa Cruz Biotechnology. All EMSA were performed several times with at least two different batches of nuclear proteins, and representative data are shown.

Transfection and Reporter Gene Assays

Reporter Gene Assays—Cells were transfected by electroporation as described (14, 15, 19). U937 cells (32 × 10^6 per sample) were transfected as follows: with 70 µg of p-TATACAT or p-B3ITATACAT; 50 µg of pSRα, HoxA10/pSRα (N312A/W313T), HoxA10/pSRα, 219–403-aa HoxA10/pSRα, 146–403-aa HoxA10/pSRα, 60–403-aa HoxA10/pSRα; and 15 µg of p-CMV-β-gal (to normalize for transfection efficiency). In some experiments, cells were also co-transfected with a vector to express the E1a viral oncprotein (26). Transfectants were incubated for 48 h at 37 °C, 5% CO₂, with and without IFN-γ (500 units/ml). Preparation of cell extracts, β-galactosidase, and chloramphenicol acetyltransferase assays were performed as described (14, 15, 19).

In other experiments, cells were transfected with 30 µg of p-(GAL4)5TKCAT and 60 µg of a vector to expression HoxA10 or various mutants as fusion proteins with the DNA-binding domain of the yeast GAL4 transcription factor. In some experiments, cells were also co-transfected with a vector to express the E1a viral oncprotein. The amount of GAL4 fusion protein expression vector used in these experiments was determined in preliminary studies in which the various HoxA10 expression plasmids were titrated to determine, for each construct, the amount of plasmid for maximal repression activity (10, 30, 60, and 120 µg). For all constructs, maximal repression activity was obtained with 60 µg of expression vector, consistent with our previous investigations with this assay (19).

Stable Transfectants—Stable U937 transfectants were generated with a vector to overexpress HoxA10 or empty vector control. Stable transfectants were selected in G418 or puromycin as described previously (14). At least three transfectant pools were tested for each construct. In some experiments, U937 cells were co-transfected with vectors to express shRNA for β3 integrin or scrambled shRNA control.

Immunoprecipitation and Western Blotting

Western Blots—Total cell lysate proteins from U937 cells or various U937 stable transfectants (50 µg) were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were serially probed with antibodies to various proteins. Antibody to β3 integrin was obtained from Chemicon-Millipore (Billericon, MA). Antibodies to tubulin, HoxA10, Syk, and phospho-Syk were obtained from Santa Cruz Biotechnology. Each experiment was performed at least three times, and representative blots are shown.

Co-immunoprecipitation Experiments—In other experiments, lysate proteins (1.5 mg) from U937 transfectants with 124–146 aa of HoxA10-Gal4 or Gal4-DBD vector control were immunoprecipitated with anti-GAL4 DNA-binding domain antibody (rabbit polyclonal from Santa Cruz Biotechnology) or irrelevant control antibody (rabbit anti-mouse IgG from Santa Cruz Biotechnology), as described above. Immunoprecipitated proteins were analyzed by Western blots and sequentially probed with antibodies to CBP or the GAL4 DNA-binding domain (all from Santa Cruz Biotechnology). This experiment was repeated twice, and representative blots are shown.

Quantitative Real Time PCR

RNA was isolated from U937 cells using the TRizol reagent, according to manufacturer’s instructions (Invitrogen). RNA was tested by denaturing gel electrophoresis to determine the integrity of the 18 S and 28 S ribosomal bands. cDNA was generated using standard techniques. Primers were designed with the software from Integrated DNA Technologies, and real time PCR was performed using SYBR green according to the “standard curve” method. Result were normalized to 18 S and actin to control for RNA abundance in various samples. In other experiments, real time PCR was performed on chromatin that co-immunoprecipitated from U937 cells with an antibody to HoxA10, the CREB-binding protein (CBP), pCAF, p300, acetyl-histone 2A, or irrelevant control. Co-immunoprecipitating chromatin was amplified with PCR primers flanking the HoxA10-binding cis element in the ITGB3 promoter. Un-precipitated chromatin was a control to normalize for DNA abundance in the various samples.

Cell Adhesion Assays

Tissue culture dishes were coated overnight at 4 °C with 20 µg/ml fibronectin. U937 cells were incubated with or without IFN-γ for 48 h, counted, and transferred to fibronectin precoated tissue culture dishes. Cells were incubated for 16 h at 37 °C, 5% CO₂. In some experiments, cells were preincubated with 20 µg/ml of antibody to β1 integrin, β3 integrin, or irrelevant control antibody (Chemicon, Temecula, CA) prior to plating on fibronectin. The dishes were washed with phos-
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Phosphate-buffered saline, and adherent cells were fixed for 40 min in 3.7% formaldehyde at room temperature. Fixed cells were stained for 40 min at room temperature with 0.1% crystal violet and solubilized in 10% acetic acid, and absorbance at 540 nm was determined as a measure of adhesion (27).

In other studies, murine bone marrow-derived, ex vivo differentiated monocytes were assayed for fibronectin adhesion. For these studies, nonadherent cells were transferred to fibronectin-coated dishes and assayed for adhesion as above.

Statistical Analysis
Statistical significance was determined by Student’s t test and analysis of variance methods using the Sigmaplot and Sigmastat software.

RESULTS
β3 Integrin Expression and β3 Integrin-dependent Fibronectin Adhesion Increased during IFNγ-induced Differentiation of U937 Myeloid Cells—The goal of these studies was to determine whether HoxA10 regulates ITGB3 promoter activity, β3 integrin expression, and phagocyte adhesion in differentiating myeloid cells. Therefore, we performed initial experiments to determine whether the U937 myelomonocytic cell line was a good model for these studies. U937 cells are a myeloid leukemia line that undergoes monocytic differentiation upon treatment with various cytokines, including IFNγ (24). IFNγ differentiation of U937 cells results in acquisition of functional characteristics of mature phagocytes over 48–72 h. These functional characteristics include respiratory burst activity, phagocytosis, and cell adhesion. In previous studies, we determined that HoxA10 protein is abundantly expressed in U937 cells and that expression is not altered by IFNγ differentiation (14, 19). Therefore, this cell line represents a reasonable model to study these events of late myeloid differentiation.

We initially determined the impact of IFNγ differentiation on β3 integrin expression. In Western blots of U937 lysate proteins, we found that abundance of β3 integrin protein increased during the 72 h of IFNγ-induced differentiation (Fig. 1A). To determine whether this increase in β3 integrin protein correlated with an increase in mRNA abundance, we determined the impact of IFNγ treatment on β3 integrin mRNA expression by real time PCR. For these studies, mRNA abundance of the respiratory burst oxidase components gp91phox and p67phox were positive controls for differentiation, and message abundance was normalized to actin and 18 S. Consistent with the protein expression data, we found that IFNγ treatment increased β3 integrin mRNA in U937 cells (Fig. 1B). These studies indicated a statistically significant increase in β3 integrin, gp91phox, and p67phox mRNA during differentiation (p < 0.01, n = 9 for all three messages).

However, these studies did not indicate a functional significance to increased β3 integrin expression during myeloid differentiation. Therefore, we investigated the role of β3 integrin in fibronectin adhesion of differentiating U937 cells. We chose to study this interaction because fibronectin is an extracellular matrix component involved in enhancing survival of hematopoietic cells (5). Interaction with fibronectin is also implicated in phagocyte rolling (8). In these studies, we found that 48 h of IFNγ differentiation significantly increased U937 cell adhesion to fibronectin (p < 0.03, n = 4) (Fig. 1C). Because both αvβ3 and α5β1 heterodimers mediate myeloid cell adhesion to fibronectin, we determined the impact of blocking antibodies to β1 or β3 integrin (or irrelevant control). We found that preincubation with anti-β1 antibody significantly decreased adhesion of untreated U937 cells to fibronectin (p < 0.001, n = 4) (Fig. 1C). However, anti-β1 antibody did not significantly impact fibronectin adhesion of IFNγ-treated cells (p = 0.2, n = 4). In contrast, preincubation with an antibody to β3 integrin did not alter adhesion of undifferentiated U937 cells to fibronectin (p = 0.5, n = 4) (Fig. 1C). However, IFNγ differentiation did not significantly alter fibronectin adhesion of U937 cells pretreated with β3 integrin antibody.

To verify the specific impact of β3 integrin on IFNγ-induced fibronectin adhesion, stable U937 transfectants were generated with a vector to express a β3 integrin-specific shRNA or scrambled shRNA control (Fig. 1D). We found that IFNγ differentiation significantly increased fibronectin adhesion of scrambled shRNA-expressing control U937 transfectants, consistent with control U937 cells (p < 0.05, n = 15) (Fig. 1C). However, expression of a β3 integrin-specific shRNA blocked IFNγ-induced fibronectin adhesion of U937 transfectants. β3 integrin-specific shRNA did not alter adhesion to fibronectin of untreated U937 cells, consistent with the blocking antibody studies above.

HoxA10 Expression Influenced β3 Integrin Expression and Fibronectin Adhesion of U937 Myeloid Cells—Therefore, we next determined the impact of HoxA10 overexpression on β3 integrin expression and fibrocnitin adhesion of U937 cells. For these studies, U937 cells were stably transfected with a vector to overexpress HoxA10 or empty control vector. Cell lysates from untreated and IFNγ-differentiated transfectants were tested by Western blot for expression of β3 integrin and HoxA10 (as a positive control). We found increased β3 integrin expression in HoxA10-overexpressing U937 transfectants in comparison with empty vector control transfectants with and without IFNγ differentiation (Fig. 2A). Neither endogenous nor overexpressed HoxA10 were altered in abundance by IFNγ differentiation of the transfectants, consistent with our previous results (14, 19).

We also investigated activation of Syk protein-tyrosine kinase, a downstream target of β3 integrin that mediates cell survival signals (8). For these studies, the blots were serially probed with antibodies to phosphorylated (activated) and total Syk. We found increased phospho-Syk in IFNγ-treated U937 transfectants overexpressing HoxA10, consistent with increased β3 integrin in these cells (Fig. 2A). In contrast, little phospho-Syk is seen in untreated HoxA10-overexpressing transfectants or control transfectants with or without IFNγ.

We also performed the reciprocal investigation to determine the impact of decreased HoxA10 protein abundance on β3 integrin expression and fibronectin adhesion. For these studies, stable U937 transfectants were generated with a vector to express a HoxA10-specific shRNA or scrambled control shRNA. Cell lysates from these transfectants were analyzed for expression of β3 integrin and HoxA10, with and
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**FIGURE 1.** β3 integrin expression and β3 integrin-dependent adhesion increased during IFNγ differentiation of U937 cells. A, IFNγ treatment of U937 cells increased β3 integrin protein expression. U937 cells were differentiated with IFNγ over a 72-h period, as indicated. Cells were harvested at various times and lysates separated by SDS-PAGE. Western blots (WB) were serially probed with antibodies to β3 integrin and tubulin (as a loading control). IFNγ treatment increased β3 integrin protein abundance over 72 h. B, IFNγ treatment of U937 cells increased β3 integrin RNA expression. U937 cells were harvested with and without 48 h of IFNγ treatment. Total cellular RNA was analyzed for abundance of β3 integrin, gp91PHOX, and p67PHOX mRNA by quantitative real-time PCR. The latter two represent messages known to be induced in differentiating U937 cells. IFNγ treatment of U937 cells significantly increased mRNA abundance of β3 integrin (indicated by *), gp91PHOX (indicated by **), and p67PHOX (indicated by #). Results were normalized to abundance of 18 S and actin mRNA to control for differences between samples in the total amount of RNA. C, β3 integrin-specific adhesion to fibronectin increased during IFNγ differentiation of U937 cells. U937 cells were tested for adhesion to fibronectin with and without IFNγ-induced differentiation. Cells were preincubated with an antibody to β3 integrin, control bovine serum albumin. Incubation with β3 integrin antibody significantly decreased fibronectin adhesion of untreated U937 cells (indicated by *) but did not impact adhesion of IFNγ-differentiated U937 cells. In contrast, incubation with β3 integrin antibody significantly decreased fibronectin adhesion of IFNγ-treated U937 cells (indicated by **) but not of undifferentiated cells. In other studies, U937 cells were stably transfected with a vector to express a β3 integrin-specific shRNA or scrambled control shRNA. Adhesion of the stable transfectants to fibronectin was determined with and without IFNγ treatment. No IFNγ-induced increase in adhesion to fibronectin was observed in cells expressing β3 integrin-specific shRNA. In contrast, expression of a β3 integrin-specific shRNA inhibited IFNγ-induced fibronectin adhesion of the transfectant cells (statistically significant difference in adhesion of differentiated transfectants is indicated by ††). D, β3 integrin protein expression was increased in U937 cells stably transfected with a vector to express a β3 integrin-specific shRNA. U937 cells were stably transfected with a vector to express a β3 integrin-specific shRNA to or scrambled control shRNA, as described above. Cells were treated with IFNγ, and total cell lysates were separated by SDS-PAGE. Western blots were serially probed with antibody to β3 integrin and tubulin (as a loading control). Decreased β3 integrin expression was seen in transfectants with the β3 integrin-specific shRNA vector in comparison with scrambled control vector.

We initially determined the effect of HoxA10 haploinsufficiency on β3 integrin expression by Western blot of monocytic lysate proteins. We found increased β3 integrin expression in both β3 integrin expression and fibronectin adhesion in differentiating myeloid leukemia cells.

**HoxA10 Deficiency Decreased β3 Integrin Expression and β3 Integrin-dependent Adhesion in Differentiating Primary Myeloid Progenitor Cells—**We also investigated the impact of HoxA10 deficiency on β3 integrin expression and fibronectin adhesion of murine bone marrow-derived monocytes. For these studies, myeloid progenitor cells were isolated from the bone marrow of HoxA10+/− mice or wild type control littermates. Progenitor cells were cultured in GM-CSF, IL3, and stem cell factor followed by differentiation to monocytes with M-CSF over 4 days (see Ref. 15).

Without IFNγ differentiation (Fig. 2B). We found that inhibition of HoxA10 expression decreased β3 integrin expression in differentiating U937 cells.

We also investigated fibronectin adhesion of U937 transfectants with either HoxA10 overexpression or HoxA10 knockdown to determine the physiologic relevance of altered β3 integrin expression in these cells. We found that HoxA10 overexpression significantly increased adhesion to fibronectin in both undifferentiated and IFNγ-treated U937 transfectants (p < 0.02, n = 6) (Fig. 2C). We also found that expression of a HoxA10-specific shRNA significantly decreased fibronectin adhesion in IFNγ-treated transfectants in comparison with transfectants with scrambled shRNA control vector (p < 0.02, n = 6) (Fig. 2C). These studies suggested that altered HoxA10 expression altered
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**FIGURE 2.** HoxA10 overexpression increased β3 integrin expression and fibronectin adhesion of U937 cells. A, HoxA10 overexpression increased β3 integrin protein abundance in U937 cells with and without IFNγ treatment. U937 cells were stably transfected with a vector to express HoxA10 or empty vector control. Lysate proteins from untransfected and IFNγ-treated transfectants were separated by SDS-PAGE, and Western blots (WB) were serially probed with antibodies to β3 integrin, HoxA10, to verify overexpression, and total and phospho-Syk (as a downstream activation target). Increased β3 integrin protein abundance was seen in HoxA10-overexpressing transfectants, with and without differentiation. This increase in β3 integrin expression was accompanied by increased phosphorylation of Syk in IFNγ-treated transfectants stably overexpressing HoxA10. Total Syk protein was the loading control in these studies. Expression of endogenous HoxA10 in control U937 transfectants was not altered by IFNγ treatment, consistent with previous results in this cell line (14). B, decreased HoxA10 expression was associated with decreased β3 integrin expression during IFNγ-induced differentiation of U937 cells. U937 stable transfectants were generated with a vector to express HoxA10-specific shRNA or scrambled control shRNA. Cell lysates from IFNγ-treated or undifferentiated transfectants were analyzed by Western blot with antibodies to HoxA10, β3 integrin, or glyceraldehyde-3-phosphate dehydrogenase (as control for loading). Inhibition of HoxA10 expression did not alter β3 integrin expression in undifferentiated transfectants. In contrast, inhibition of HoxA10 expression was associated with decreased IFNγ-induced β3 integrin expression in differentiating U937 cells. β3 integrin expression was increased, and HoxA10 expression was not altered by IFNγ-treatment of transfectants with control, scrambled shRNA, consistent with results of the experiments above. C, HoxA10 overexpression increased and HoxA10 knockdown decreased adhesion of U937 cells to fibronectin. U937 cells were stably transfected with a vector to express HoxA10 or empty vector control, or a HoxA10-specific shRNA or scrambled shRNA control. Adhesion to fibronectin was determined with and without IFNγ-treatment of the transfectants. HoxA10 overexpression significantly increased fibronectin adhesion of U937 transfectants without (indicated by *) and with (indicated by **) IFNγ treatment, consistent with the effect of HoxA10 expression on β3 integrin expression. In contrast, adhesion of IFNγ-treated transfectants expressing HoxA10-specific shRNA was significantly less than control, scrambled shRNA transfectants (indicated by #).

*ex vivo* differentiated wild type monocytes in comparison with HoxA10 haploinsufficient monocytes (Fig. 3A). These results were consistent with the studies with HoxA10 knockdown in U937 cells.

We also assayed these *ex vivo* differentiated cells for fibronectin adhesion. In these studies, we found that *ex vivo* differentiated wild type monocytes demonstrated significantly more fibronectin adhesion in comparison with HoxA10+/− monocytes (p < 0.02, n = 4) (Fig. 3B). These studies further suggested a role for HoxA10 in β3 integrin expression and cell adhesion in differentiating myeloid cells.

Increased Fibronectin Adhesion of HoxA10-overexpressing U937 Cells Was β3 Integrin-dependent—To determine whether increased fibronectin adhesion of HoxA10-overexpressing U937 cells was dependent on β3 integrin, stable transfectants were generated with a vector to overexpress HoxA10 or control vector and a vector to express β3 integrin-specific shRNA or scrambled shRNA control. Cell lysates from IFNγ-treated transfectants were tested by Western blot to verify inhibition of β3 integrin expression by β3 integrin-specific shRNA in HoxA10-overexpressing transfectants (Fig. 4A).

These stable transfectants were assayed for fibronectin adhesion assays with and without IFNγ differentiation. We found that HoxA10 overexpression significantly increased fibronectin adhesion of U937 cells co-transfected with a vector to express scrambled control shRNA, with and without IFNγ differentiation, similar to the experiments in the section above (p < 0.001, n = 4) (Fig. 4B). In contrast, HoxA10 overexpression did not significantly increase fibronectin adhesion in untreated cells...
co-transfected with the β3 integrin-specific shRNA expression vector ($p = 0.7, n = 4$). Additionally, expression of β3 integrin-specific shRNA blocked IFNγ-induced fibronectin adhesion of HoxA10 overexpressing stable transfectants (Fig. 4B). These results suggested that β3 integrin expression was necessary for HoxA10-induced fibronectin adhesion.

**HoxA10 Activated a Cis Element in the ITGB3 Promoter**—We next investigated the impact of HoxA10 on the activity of the ITGB3 promoter. Previous studies demonstrated *in vitro* interaction of HoxA10 with a Hox/Pbx-binding consensus sequence in the ITGB3 5′ flank by EMSA using nuclear proteins from endometrial carcinoma cells (10). To determine whether this sequence functions as a HoxA10-activated cis element in myeloid cells, we generated an artificial promoter construct with multiple copies of the putative cis element linked to a minimal pro-
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HoxA10/Pbx2 Heterodimer Binds the ITGB3 Promoter in a Multiprotein Complex—Because this ITGB3 cis element was homologous to derived consensus sequences for DNA binding of HoxA10/Pbx heterodimers, we investigated whether HoxA10 partners with a Pbx protein to bind this cis element. In our previous studies of the CYBB and NCF2 genes, we found that HoxA10 interacted with negative cis elements in these genes in vitro and in vivo in assays with undifferentiated U937 cells. During IFN-γ differentiation, HoxA10 binding to these negative CYBB and NCF2 cis elements decreased, resulting in decreased repression. However, the functional assays above suggested that HoxA10 bound to the ITGB3 cis element in IFN-γ-treated cells and activated transcription during differentiation. This suggested the possibility that differentiation increased HoxA10 binding to the ITGB3 cis element, the opposite of the effect on HoxA10 binding to the CYBB and NCF2 genes.

Therefore, we performed EMSA to determine the impact of IFN-γ differentiation on HoxA10 interaction with the ITGB3 promoter cis element. For these studies, nuclear proteins were isolated from U937 cells, with or without 48 h of IFN-γ treatment, and used in binding assays with a radiolabeled probe representing the ITGB3 cis element (bp −1973 to −1933). As a control for IFN-γ-induced U937 differentiation, EMSA were also performed with a probe representing the negative CYBB cis element (bp −94 to −134). In these studies, we found that IFN-γ treatment of U937 cells increased binding of a low mobility protein complex to the ITGB3 cis element (Fig. 5B). In additional studies, we determined that this complex represented specific protein binding to the Hox/Pbx consensus sequence in the probe (not shown; consistent with Ref. 10). Consistent with our previous results, IFN-γ treatment decreased binding of a low mobility protein complex of similar mobility to the CYBB probe. We have shown previously that this complex included HoxA10 and Pbx1.

Therefore, we performed additional experiments to determine whether the low mobility protein complex, which bound to the ITGB3 cis element, included HoxA10 and/or a Pbx protein. For these studies, nuclear proteins from IFN-γ-treated U937 cells were preincubated with antibodies to HoxA10, Pbx1, Pbx2, or irrelevant control antibody and used in EMSA with the ITGB3 probe. We found that anti-HoxA10 antibody disrupted binding of the protein complex to this probe (Fig. 5C). Additionally, we found that antibody to Pbx2, but not Pbx1, also disrupted this complex. These results suggested that the ITGB3 cis element was activated by a HoxA10/Pbx2 heterodimer.

In these studies, we found that HoxA10 bound to the ITGB3 cis element in vivo in undifferentiated U937 cells. These results further suggested the hypothesis that HoxA10 activated the ITGB3 promotor in differentiating myeloid cells.

The Pbx Interaction Domain Is Not Identical to the HoxA10 Activation Domain—For some previously described Hox/Pbx target genes, the Pbx protein recruited transcriptional co-activator or co-repressor proteins to Hox/Pbx binding cis elements (18). The Pbx protein was therefore essential for modulation of transcription of such target genes. Therefore, we investigated whether HoxA10 activation of the ITGB3 promoter cis element required interaction with a Pbx protein. To do this, we used a previously described form of HoxA10 with mutation in the Pbx binding “hexapeptide” (referred to as (N312A/W313T) HoxA10) (28). Mutation of these residues was demonstrated previously to prevent interaction of HoxA10 with Pbx proteins but not HoxA10-DNA binding (15, 19, 28).

We co-transfected U937 cells with the reporter vector containing multiple copies of the ITGB3 cis element or empty vector control and a vector to overexpress (N312A/W313T) HoxA10. Reporter gene activity was determined for IFN-γ-treated and -untreated U937 transfectants, and we compared it with transfectants with wild type HoxA10 or empty control vector (Fig. 5A). We found that activation of the ITGB3 cis element was not significantly different in transfectants with wild type versus (N312A/W313T) HoxA10, with or without IFN differentiation (p > 0.2, n = 7). In control experiments, neither expression of wild type nor (N312A/W313T) HoxA10 influenced expression of empty pTATACAT control reporter vector. Consistent with previous results, wild type and (N312A/W313T) HoxA10 were equivalently expressed in U937 transfectants (not shown) (19).
These results suggested that the Pbx interaction domain was not identical to the HoxA10 activation domain. Therefore, we pursued identification of the domain involved in HoxA10 activation of ITGB3 transcription during myeloid differentiation.

HoxA10 Activates Transcription via a "PQ-like" Domain—We employed two different reporter assays to identify the HoxA10 activation domain. For the first assay system, we expressed various HoxA10 truncation mutants as fusion proteins with the DNA-binding domain of the yeast Gal4 transcription factor (referred to as Gal4-DBD). These HoxA10-Gal4-fusion proteins were assayed for activity by co-transfecting U937 cells with a vector to express the fusion protein and a reporter construct containing multiple copies of a Gal4-DNA-binding site linked to a minimal promoter. The advantage to this approach was that HoxA10 peptide sequences could be tested in isolation from the DNA-binding homeodomain. In the second set of assays, we tested the ability of HoxA10 truncation mutants to activate transcription from the cis element in the ITGB3 gene using the minimal promoter/reporter constructs discussed above. The advantage to this approach was that it permitted functional assessment of the putative activation domain in the context of a genuine target gene.

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We initially compared the effect on reporter activity of wild type HoxA10-Gal4 fusion protein versus a series of HoxA10 truncation mutant fusion proteins (1–112-aa HoxA10-Gal4, 1–179-aa HoxA10-Gal4, and 1–219-aa HoxA10-Gal4) versus control Gal4-DBD. Consistent with our previous results, expression of HoxA10-Gal4 induced significant repression of this reporter construct in U937 transfecants in comparison with control Gal4-DBD ($p < 0.001, n = 8$) (Fig. 6A) (19). In contrast, we found that expression of either the 1–179-aa HoxA10-Gal4 fusion protein or the 1–219-aa HoxA10-Gal4 fusion protein was associated with significant activation of this reporter construct in U937 transfecants (about 200% increase, $p < 0.05, n = 5$) (Fig. 6A). However, the 1–112-aa HoxA10-Gal4 fusion protein did not significantly impact expression from this reporter construct in comparison with control Gal4-DBD ($p = 0.8, n = 7$). Based on these results, we concluded there was a "cryptic" activation domain between HoxA10 amino acids 112 and 179.

Additional truncation mutants were generated to further define this HoxA10 domain, including amino acids 102–146, 124–146, and 145–219 of HoxA10. We found that reporter expression in transfecants expressing either 102–146-aa HoxA10-Gal4 or 124–146-aa HoxA10-Gal4 was not significantly different from transfecants with 1–179-aa HoxA10-Gal4 or 1–219-HoxA10-Gal4 ($p = 0.51, F = 0.78, n = 7$) (Fig. 6A). In contrast, reporter activity from the GAL4-binding domain construct in transfecants expressing 145–219-aa HoxA10-Gal4 was significantly less than reporter activity in transfecants with any of these other constructs ($p < 0.005, F = 4.2, n = 7$). These studies suggested that the HoxA10 activation domain is between amino acids 124 and 146.

Based on these results, we generated a series of N-terminal HoxA10 truncation mutants to assay for activation of the ITGB3 cis element, as described above. These truncation mutants were engineered to include the genuine HoxA10 Kozak consensus sequence, an ATG start codon, and the DNA-binding homeodomain (amino acids 319–403). U937 cells were co-transfected with the ITGB3 cis element-containing reporter construct and a vector to express wild type HoxA10, 60–403-aa HoxA10, 146–403-aa HoxA10, 219–403-aa HoxA10, or empty vector control. Reporter gene assays were performed with and without IFNγ differentiation. Transcriptional activation of the ITGB3 cis element-containing reporter construct was significantly greater in untreated transfecants with wild type or 60–403-aa HoxA10 in comparison with control vector transfecants (indicated by *). ITGB3 cis element activation was also significantly greater in IFNγ-treated transfecants overexpressing wild type or 60–403-aa HoxA10 in comparison with control (indicated by **). In contrast, reporter activity from the ITGB3 cis element is significantly repressed by expression of 145–403-aa HoxA10 or 219–403-aa HoxA10 in undifferentiated transfecants (indicated by #). Repression of ITGB3 transfectants by 145–403-aa HoxA10 or 219–403-aa HoxA10 is relieved by IFNγ treatment of the transfecants. None of these proteins influence reporter expression from control pTATA-CAT vector. HoxA10 activation domain is homologous to PQ domains found in transcriptional activators. The HoxA10 124–146-aa sequence was used to search a peptide data base for homology to known transcription factors. This domain has homology to previously described PQ domains in other proteins with transcriptional activation function such as the E1a-binding protein and bromodomain containing protein 4.

We found that activation of the ITGB3 cis element was not significantly different in transfecants overexpressing wild type HoxA10 versus 60–403-aa HoxA10, with and without IFNγ treatment ($p = 0.4, n = 7$) (Fig. 6B). In contrast, both 146–203-aa HoxA10 and 219–403-aa HoxA10 significantly
repressed reporter expression from the ITGB3 cis element-containing construct in comparison with control in untreated U937 transfectants, although this repression activity was abolished by differentiation. Indeed, reporter activity from the ITGB3 cis element-containing reporter construct in IFNγ-treated transfectants with 146–203-aa HoxA10 or 219–403-aa HoxA10 was not significantly different from reporter activity in vector control transfectants ($p = 0.74, F = 0.3, n = 4$). None of these proteins influence expression from empty pTATACAT control vector, with or without IFNγ differentiation.

These studies suggested that the HoxA10 activation domain was not identical to the Pbx interaction domain but resided between amino acids 124 and 146. Therefore, we were interested in further examining this peptide sequence to identify homology with known functional domains in other proteins. To do this, we performed a database search using the National Center for Biotechnology Information website (National Institutes of Health, National Library of Medicine). We found that this HoxA10 peptide sequence had homology to "PQ" domains in other transcription factors, including the E1a-binding protein p300 (29) and bromodomain containing protein 4 (30) (Fig. 6C). These proteins activate gene transcription via interaction with transcriptional co-activators. Therefore, we next investigated whether HoxA10 aa 124–146 recruited transcriptional co-activators as a potential mechanism for transcriptional activation.

E1a Expression Inhibits HoxA10 Activation of the ITGB3 Cis Element—Based on homology of the HoxA10 activation domain with the PQ domain in the E1a-binding protein, we hypothesized that this domain might recruit E1a-interacting transcriptional co-activators to the ITGB3 promoter. E1a is a viral oncoprotein that interacts with the transcriptional co-activators, including the CREB-binding protein (CBP), p300, and pCAF (31). E1a interacts with specific domains in these co-activators and prevents interaction with transcription factors, thereby blocking gene transcription. Therefore, we tested the impact of E1a expression on the HoxA10 activation domain.

For these studies, U937 cells were co-transfected with a vector to express HoxA10-Gal4, 124–146-aa HoxA10-Gal4, or control Gal4-DBD vector, a vector to express E1a or empty control vector, and a reporter vector with multiple copies of a Gal4-binding cis element linked to a minimal promoter. We found that E1a expression significantly decreased activation of the reporter construct by 124–146-aa HoxA10-Gal4 ($p < 0.0001, n = 9$) (Fig. 7A). Indeed, reporter expression in transfectants expressing 124–146-aa HoxA10-Gal4 + E1a was not significantly different from reporter expression in transfectants with empty Gal4-DBD vector control ($p = 0.98, n = 9$). E1a expression also significantly increased repression activity of HoxA10-Gal4 in this assay ($p = 0.01, n = 9$). These results suggested that expression of E1a in U937 cells interfered with interaction of a transcriptional co-activator and the HoxA10 PQ-like domain.

We were also interested in determining the impact of E1a expression on HoxA10 activation of the ITGB3 cis element. For these studies, U937 cells were co-transfected with the minimal promoter/reporter vector with multiple copies of the ITGB3 cis element (B3ITATACAT) or empty control vector (pTATACAT), a vector to express HoxA10 or empty vector control, and a vector to express E1a or empty vector control. Reporter expression was determined with and without IFNγ differentiation of the transfectants.

In these studies, we found that E1a expression inhibited HoxA10 activation of the ITGB3 cis element in both untreated and IFNγ-differentiated transfectants ($p \leq 0.01, n = 4$) (Fig. 7B). Specifically, reporter activity in these transfectants was not significantly different from transfectants with 3ITATACAT without HoxA10. In control experiments, HoxA10 did not activate the pTATACAT control vector, with or without IFNγ, with or without E1a.

The HoxA10 PQ-like Domain Recruited CBP to the ITGB3 Cis Element—The results of our functional studies suggested that HoxA10 activation of the ITGB3 cis element required interaction with a transcriptional co-activator. Detecting interaction of transcriptional co-activator proteins with DNA-bound transcription factor complexes can be difficult because these large complexes do not migrate well into acrylamide gels. Therefore, we performed chromatin co-immunoprecipitation using U937 cells with and without IFNγ differentiation. For these studies, proteins were cross-linked to chromatin in vivo, and cell lysates were immunoprecipitated with an antibody to HoxA10 or the transcriptional co-activators CBP, pCAF, p300, or irrelevant control antibody. Immunoprecipitates were quantitated by real time PCR with primers flanking the HoxA10-activated cis element in the ITGB3 promoter (generating an ~90 bp product). Results were normalized to nonprecipitated chromatin to control for differences in DNA abundance between the samples. In these studies, we found that in vivo HoxA10 binding to this cis element increased significantly upon IFNγ differentiation of U937 cells ($p < 0.02, n = 6$) (Fig. 7C). We also found that IFNγ differentiation significantly increased in vivo CBP binding to the ITGB3 cis element ($p < 0.02, n = 4$). In contrast, there was no significant co-precipitation of ITGB3 cis element chromatin in experiments with antibody to pCAF or p300 in comparison with control, irrelevant antibody (not shown).

Because CBP has histone acetyltransferase activity, recruitment to the ITGB3 cis element by HoxA10 would be expected to increase acetylation of locally bound histones, decreasing chromatin compaction, and favoring transcription. Therefore, we used chromatin immunoprecipitation to analyze binding of acetylated histone 2A (H2A) to the ITGB3 cis element during IFNγ differentiation. This antibody was chosen because histone H2 is one of the preferred substrates for histone acetyltransferase activity of CBP. In these studies, we found that IFNγ differentiation significantly increased abundance of ITGB3-bound acetyl H2A ($p < 0.001, n = 4$).

These studies suggested that HoxA10 recruited CBP to the ITGB3 cis element, and this resulted in transcriptional activation. Therefore, we performed additional investigations to determine whether CBP interacted with the HoxA10 PQ-like activation domain. For these studies, U937 cells were transfected with a vector to express 124–146-aa HoxA10-Gal4 or control Gal4-DBD. Lysate proteins from these transfectants were immunoprecipitated under nondenaturing conditions with an antibody to the Gal4-DBD or an irrelevant control antibody. Precipitated proteins were separated by SDS-PAGE, and
Western blots were serially probed with antibodies to CBP and the Gal4-DNA-binding domain (as an expression control).

Consistent with our hypothesis, we found that CBP specifically co-precipitated with the HoxA10-PQ domain (Fig. 7D). We also found that this association was greater in assays with proteins from the differentiated transfectants. Control blots probed with the Gal4-DBD antibody demonstrated that 124–146-aa HoxA10-Gal4 and control Gal4-DBD were equivalently expressed, and neither protein was altered in abundance by IFNγ-treatment of the transfectants.

**DISCUSSION**

In these studies, we found that HoxA10 was required for increased ITGB3 transcription and β3 integrin expression dur-
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ing myeloid differentiation. We also found that this increase in β3 integrin expression was functionally relevant to the increase in fibronectin adhesion observed in differentiating myeloid cells. Although HoxA10 bound to an ITGB3 cis element as a heterodimer with Pbx2, we found that interaction with Pbx2 was dispensable for HoxA10 activation of this cis element. Consistent with this, we found that the HoxA10 activation domain was not identical to the Pbx-interacting hexapeptide but was a novel domain, not conserved in other Abd HoxA proteins. This domain was homologous to PQ domains that mediate protein–protein interactions in other transcriptional activator proteins. In additional studies, we found that the HoxA10 PQ-like domain recruited CBP to the ITGB3 promoter, in vitro and in vivo. Therefore, these studies identified ITGB3 as a genuine target gene activated by HoxA10 in differentiating myeloid cells, determined that HoxA10 expression levels were functionally significant for fibronectin adhesion of differentiating myeloid cells, identified a unique HoxA10 activation domain, and determined that this domain recruited a transcriptional co-activator protein to a target gene promoter.

The Abd HoxA proteins, including HoxA10, are known to play key regulatory roles in embryogenesis and hematopoiesis (reviewed in Ref. 32). Despite this, relatively few genuine target genes have been identified for these homeodomain proteins. The issue of Hox target genes is of some complexity, because Hox proteins can either activate or repress transcription of various genes, depending upon context. Consistent with this, both activation and repression target genes have been described for HoxA10. For example, previous studies identified a sequence in the ITGB3 promoter that was activated by overexpressed HoxA10 in epithelial carcinoma cells (10). In contrast, we found that the CYBB and NCF2 genes were repressed by HoxA10 in undifferentiated myeloid cells (14, 15). Because expression of β3 integrin increases during myelopoiesis, we investigated ITGB3 transcription as a potential model for transcriptional activation by HoxA10 during myeloid differentiation. During the course of these studies, we determined that HoxA10 activated ITGB3 transcription during myelopoiesis. Therefore, these studies identified a target gene that is activated by HoxA10 and is involved in the functional activities of mature phagocytes.

In these studies, we found that fibronectin adhesion of U937 cells undergoing IFN-γ differentiation is dependent on HoxA10-induced β3 integrin expression. We also found that HoxA10 overexpression increased Syk activation in differentiating myeloid cells. Because Syk kinase activity is associated with generation of cell survival signals (8), these results suggested a mechanism by which HoxA10 overexpression might antagonize apoptosis of differentiating myeloid cells. This is of potential importance because HoxA10 overexpression has been associated with poor prognosis in human acute myeloid leukemia and induces a myeloproliferative disorder in murine models.

Although the various functions of β3 integrin in mature phagocytes are well established, mechanisms that regulate β3 integrin activity are not. Myeloid cell adhesion via α5β1 integrin is regulated by signal-dependent changes in integrin glycosylation, specifically in α2–6-sialylation of the β1 subunit (27, 33). However, no similar modification of β3 integrin occurs (see Ref. 34). Our studies identified an impact of IFN-γ-induced myeloid differentiation on β3 integrin expression via increased ITGB3 transcription. These results suggested that cytokine-stimulated expression may regulate β3 integrin function in differentiating myeloid cells or during the inflammatory response.

Although we found that IFN-γ differentiation of U937 cells increased β3 integrin-dependent fibronectin adhesion, this stimulation did not influence β1 integrin–dependent adhesion. In contrast, stimulation of U937 cells with phorbol myristate acetate increased fibronectin adhesion mediated by β1 integrin (27). The system of integrin regulation and function is obviously complex, involving post-translational modification and gene transcription. The importance of such fine-tuning of integrin expression and function in differentiating myeloid cells is not known but will be of considerable interest for understanding phagocyte function.

In current studies, we determined that HoxA10 bound the ITGB3 promoter as a heterodimer with the homeodomain protein Pbx2. However, in further studies, we found that interaction with Pbx2 was not essential for HoxA10 activation of the ITGB3 cis element. DNA-bound Hox/Pbx heterodimers have been shown to activate or repress some target genes via the ability of the Pbx partner to recruit transcriptional co-activator or co-repressor proteins (18). In such cases, the Hox partner is hypothesized to select the specific binding site for the heterodimer. In the current studies, we identified a novel HoxA10 activation domain that recruited a transcriptional co-activator protein to the ITGB3 promoter in a Pbx–independent manner. Although Pbx2 may increase the affinity of HoxA10 to the ITGB3 cis element, this effect was not apparent in our transfection studies. It is possible that increased HoxA10 binding affinity, because of interaction with Pbx2, may be relevant at endogenous HoxA10 levels but not in cells overexpressing HoxA10.

In previous studies, we determined that the CYBB and NCF2 genes decreased during myeloid differentiation on ITGB3 expression. These results suggested that cytokine-stimulated expression may regulate β3 integrin function in differentiating myeloid cells or during the inflammatory response. In contrast, interaction of HoxA10 with the repressor cis element in the CYBB and NCF2 genes decreased during myelopoiesis (14, 15). Decreased HoxA10 binding to the

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CYBB and NCF2 cis elements was related to inhibitory phosphorylation of tyrosine residues in the homeodomain in response to differentiating cytokines (36). The mechanism for increased HoxA10 interaction with the positive ITGB3 cis element in response to differentiation is not known but is a topic of current investigations in the laboratory.

Of additional interest are mechanisms involved in regulating activity of the HoxA10 activation domain versus the HoxA10 repression domain at various stages of differentiation or for various target genes. It is possible that function of these two domains is regulated by HoxA10 post-translational modification in response to cytokines. Alternatively, the presence of partner proteins might dictate whether the HoxA10 activation or repression domain is functionally dominant at a particular differentiation stage. Another possibility is that various binding sites alter HoxA10 conformation differently, favoring activity of either the activation or repression domain. Superficial examination of the CYBB and NCF2 versus ITGB3 cis elements did not provide an immediate explanation for variation in differentiation stage-specific interaction of HoxA10 with these genes or differential function. All three cis element have homology to previously described derived Hox/Pbx binding consensus sequences (37). Identification of additional HoxA10 target genes may clarify this issue.

Despite the importance of Abd HoxA proteins to normal and malignant myelopoiesis, the function and regulation of these proteins is poorly understood. In these studies, we identified a HoxA10 target gene that is activated during myeloid differentiation. The function and expression of this target gene as a model system to identify the HoxA10 activation domain and the mechanism by which this domain influences transcription. Such studies of Abd HoxA proteins may shed light on the oncogenic effect of overexpression of these proteins in myeloid malignancies.

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