The NFY Transcription Factor Inhibits von Willebrand Factor Promoter Activation in Non-endothelial Cells through Recruitment of Histone Deacetylases*

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Human von Willebrand factor (VWF) gene sequences +155 to +247 contain cis-acting elements that contribute toward endothelial specific activation of the VWF promoter. Analyses of this region demonstrated the presence of a GATA-binding site that is necessary for the promoter activation in endothelial cells. We have reported recently the presence of a novel NFY-binding sequence in this region that does not conform to the consensus NFY-binding sequence CCAAT. NFY was shown to function as a repressor of the VWF promoter through interaction with this novel binding site. Here we report that the NFY interacts with histone deacetylases (HDACs) in a cell type-specific manner and recruits them to the VWF promoter to inhibit the promoter activity in non-endothelial cells. Analyses of the acetylation status of histones in the chromatin region containing the VWF promoter sequences demonstrated that these sequences are associated with acetylated histone H4 specifically in endothelial cells. It was also demonstrated that HDACs are specifically recruited to the same chromatin region in non-endothelial cells. We also demonstrated that GATA6 is the GATA family member that interacts with the VWF promoter and that GATA6 is associated with NFY specifically in non-endothelial cells. We propose that NFY recruits HDACs to the VWF promoter, which may result in deacetylation of GATA6 as well as of histones in non-endothelial cells, thus leading to promoter inactivation. In endothelial cells, however, association of HDACs, NFY, and GATA6 is interrupted potentially through endothelial cell-specific signaling/mechanism, thus favoring the balance toward acetylation and activation of the VWF promoter.

The important role of endothelial cells in many physiological and pathophysiological processes has made the regulation of gene expression in these cells a subject of intensive investigations (1). A number of endothelial cell-specific promoters have been identified (2). Several trans-acting factors including GATA2 (3, 4), GATA3, GATA6 (5), Ets (6, 7), SCL/Tal-1 (8), SP1 (9), oesf (10), Oct1 (11, 12), and NF1 (13) in various combinations have been shown to participate in the regulation of these endothelial specific promoters. Although some of these tran-

* This work was supported by National Institute of Health Research Grants HL-54678 and HL-67729 (to N. J.) and American Heart Association Grant AHA2256086T (to Y. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org

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Analyses of the endothelial specific regulation of the von Willebrand factor (VWF) promoter have resulted in characterization of several trans-acting factors that participate in regulation of the activity of this promoter. An Et1 and a member of the GATA family of trans-acting factors were shown to function as activators while Oct1 and NF1 were shown to function as repressors of the VWF promoter (7, 13, 11, 26). Recently, we have demonstrated (27) that the NFY transcription factor functions as both an activator and a repressor of the VWF promoter activity. This dual repressor/activator function of NFY appears to be modulated through its differential binding sequences. Two distinct DNA sequences with no homologies are used as binding sites for NFY in the VWF promoter. NFY functions as an activator when interacting with the sequence CCAAT (previously reported NF consensus binding site) at position −18 in the VWF promoter, whereas it functions as a repressor when interacting with a novel binding sequence (sequences +226 to +234) located in the first exon (27).

Based on these results, we have hypothesized that the function of NFY may be dependent on the cofactors that interact with this ubiquitous trans-acting factor and thus modulate its trans-acting function as either an activator or a repressor. In this report, we demonstrated that the function of NFY as a repressor is mediated through cell type-specific recruitment of HDACs to the VWF promoter, resulting in the promoter inactivation in non-endothelial cells. We also identified GATA6 as the member of the GATA trans-acting factor family that interacts with the VWF promoter and demonstrates a cell type-specific association of GATA6 and NFY. We hypothesized that these cell-specific associations of DNA-binding trans-acting factors and cofactors provide a molecular basis for determining the endothelial specific activation pattern of the VWF gene expression.

MATERIALS AND METHODS

Cell Culture, Transfection, and Plasmid Generation—HEK293 cells and human umbilical vein endothelial cells (HUVEC) were grown and maintained as described previously (27, 28). Generation of plasmids HGH-1, HGH-1K, and HGH-1KY and transfection of HEK293 cells maintained as described previously (27, 28). Generation of plasmids and human umbilical vein endothelial cells (HUVEC) were grown and prepared by the method of Schreiber et al. (26). Nuclear extract (12°C) were pre-cleared and precleared and incubated on ice for 20 min, followed by the addition of Nonidet P-40 (final concentration of 0.5%) and brief vortexing. Nuclear pellets obtained by brief centrifugation were resuspended in buffer 4 (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1.0 m M PMSF, and 100 ng/ml leupeptin and aprotinin) and incubated on ice for 15 min. The cross-linked chromatin was subjected to sonication using VibraCell™ (Sonics and Material Inc., Danbury, CT) to obtain DNA fragments of ~200 bp. Fragmented chromatin was pelleted by brief centrifugation, and samples corresponding to ~106 cells were diluted 10 times in buffer C (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.0), 167 mM NaCl, 1 mM PMSF, and 100 ng/ml leupeptin and aprotinin). Prior to immunoprecipitation the samples were pre-cleared with the addition of 80 μl of A/G protein for 2 h at 4°C. An aliquot of the pre-cleared chromatin (10 μl) was saved for analysis of total input, and the remaining samples were immunoprecipitated with 5 μg of appropriate antibodies or no antibody. The immunoprecipitated chromatin was washed sequentially with wash buffers 1, 2, 3, and 4 (wash buffer 1: 150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40; wash buffer 2: 0.1% SDS, 1% Nonidet P-40, 50 mM Tris (pH 8.0), 5 mM NaCl; wash buffer 3: 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris (pH 8.1), 1 mM EDTA; and wash buffer 4: 10 mM Tris (pH 8.1), 1 mM EDTA). The chromatin was eluted by a 15-min incubation in 150 μl of fresh buffer E (1% SDS, 50 mM NaHCO3). To reverse cross-linking, the samples were incubated at 65°C for 4 h in a buffer containing 200 mM NaCl and 1 μg of RNase A followed by treatment with proteinase K and ethanol precipitation. The pellets were collected by microcentrifugation, resuspended in 20 μl of H2O, and subjected to PCR analysis. The PCRs were performed with appropriate primer pairs. The human VWF promoter region from −30 to +155 was amplified with the following primers: primer 1, 5′-ATCAAAAGAGGAGCAATCCCTGCTGGTGCGG-3′; and primer 2, 5′-GGTGGCCTGCTAATCGAACGGAAGACGACG-3′ (according to the VWF sequence +127 to +156), and HGH-2, 5′-AGTTGGGGAGGGTGGCATGAGGAGGACGAC-3′; primer 3, 5′-CCCCCTCGAAATAGGGCTGCGCTACTGCAAG-3′; primer 3′, 5′-CTCCTCCTAGGAGAGGTGGCGCTATTCCAAG-3′. The VWF-HGH plasmids in stably transfected cells were amplified with HHIT, 5′-TACGACCATGTTGCATTGCTTCAGGCAAGACA-3′ (according to the human growth hormone gene starting from the 1 position of the human growth hormone gene (provided by Nichols Institute, San Juan Capistrano, CA). The VWF promoter in all VWF-HGH plasmid was cloned immediately upstream of the +1 position of the human growth hormone gene.

The VCM-specific primers are VCM-3T, 5′-GCCCACTCTATTGTCC-3′, and VCM-4B, 5′-TCCCTTCTCTGC-3′. PCR reactions were performed with appropriate primer pairs. The human VWF promoter region from −30 to +155 was amplified with the following primers: primer 1, 5′-ATCAAAAGAGGAGCAATCCCTGCTGGTGCGG-3′; and primer 2, 5′-GGTGGCCTGCTAATCGAACGGAAGACGACG-3′ (according to the VWF sequence +127 to +156), and HGH-2, 5′-AGTTGGGGAGGGTGGCATGAGGAGGACGAC-3′; primer 3, 5′-CCCCCTCGAAATAGGGCTGCGCTACTGCAAG-3′; primer 3′, 5′-CTCCTCCTAGGAGAGGTGGCGCTATTCCAAG-3′. The human VWF promoter region from −30 to +155 was amplified with the following primers: primer 1, 5′-ATCAAAAGAGGAGCAATCCCTGCTGGTGCGG-3′; and primer 2, 5′-GGTGGCCTGCTAATCGAACGGAAGACGACG-3′ (according to the VWF sequence +127 to +156), and HGH-2, 5′-AGTTGGGGAGGGTGGCATGAGGAGGACGAC-3′; primer 3, 5′-CCCCCTCGAAATAGGGCTGCGCTACTGCAAG-3′; primer 3′, 5′-CTCCTCCTAGGAGAGGTGGCGCTATTCCAAG-3′. 

RESULTS

The VWF Promoter Activity Is Associated with Acetylated Histone H4—We have demonstrated recently (27) that NFY transcription factor functions both as an activator and a repressor of the VWF promoter activity through two distinct binding sequences. Because NFY is shown to associate with cofactors that function as HATs (30, 32, 33), we hypothesized that the dual function of NFY may be modulated through recruitment of HATs and HDACs depending on its binding sequence. To test this hypothesis, we carried out ChIP experiments to first de-
NFY and acetylated histone H4 are associated with the VWF promoter. A schematic representation of the VWF promoter with trans-acting factor binding sites is shown. Arrows show the positions of the PCR primers used in ChIP analyses. B, ChIP analyses using anti-NFY-A antibody were carried out. The immunoprecipitated chromatin from HUVEC and HEK293 cells was subjected to PCR analyses using primers that amplify the sequences +155 to +247 for detecting the VWF promoter and specific primers (described under “Materials and Methods”) to detect the actin promoter (as control) sequences. The input panel represents the non-immunoprecipitated chromatin fraction used as template for PCR. The amplified VWF and actin fragments were 92 and 160 bp, respectively. C, chromatin from HUVEC and HEK293 cells was immunoprecipitated with no antibody (Ab), anti-NFY-A, anti-acetylated histone H3 (AH3), and anti-acetylated histone H4 (AH4) antibodies. The immunoprecipitated chromatin was subjected to PCR using primers that amplify the sequences −30 to +155 of the VWF promoter. The amplified PCR fragments were analyzed on 1.5% agarose gels. The input panel represents the non-immunoprecipitated chromatin fraction used as template for PCR.

terminate whether NFY interacts with the VWF promoter in the context of chromatin in vivo and whether the VWF promoter activity is associated with the status of histone acetylation.

For these analyses, we carried out ChIP experiments of the endogenous VWF promoter in human umbilical vein endothelial cells (HUVEC) and HEK293 cells. These two cell types were chosen as models of human VWF expressing (HUVEC) and non-expressing (HEK293) cell types.

To determine directly whether NFY interacts with chromatin containing the VWF promoter, we used antibodies that specifically recognize the NFY-A subunit of NFY complex to immunoprecipitate native chromatin from these two cell types. We then determined the presence of the VWF promoter fragments in the immunoprecipitated fractions using PCR analysis with the VWF-specific primers that amplify the +155 to +247 sequences of the VWF promoter (Fig. 1A). These sequences contain the novel NFY-binding site that functions as a repressor, although precipitated chromatin fragments may also include the VWF promoter sequences that contain the upstream CCAAT-NFY-binding site. Primers that specifically recognize the actin promoter sequences were used as control.

The results demonstrated that NFY-A immunoprecipitated chromatin in both HUVEC and HEK293 cells was specifically amplified with the VWF-specific primers but not with the actin-specific primers (Fig. 1B, upper panel). Both the actin and the VWF-specific primers amplified the appropriate gene fragments from non-immunoprecipitated chromatin input, demonstrating PCR efficiency (Fig. 1B, lower panel). The fragment sizes amplified by the VWF and the actin primers were 92 and 160 bp, respectively. These data demonstrated that NFY interacts with endogenous VWF promoter but not the actin promoter in the context of chromatin in both HUVEC and HEK293 cells (Fig. 1B). To demonstrate directly whether the active VWF promoter is associated with acetylated histones, we used antibodies that specifically recognize acetylated histone H3 and acetylated histone H4 to immunoprecipitate native chromatin from these two cell types. We then determined the presence of the VWF promoter fragment in the immunoprecipitated fractions using PCR analysis with the VWF-specific primers that amplify a 185-bp fragment corresponding to the sequences −30 to +155 region of the VWF promoter (Fig. 1A). We also included anti-NFY-A antibody in these analyses to confirm the interaction of NFY with the VWF promoter sequences with these set of primers. Immunoprecipitated samples in absence of antibodies were used as control for PCR analysis.

The results of these analyses confirmed the interaction of NFY transcription factor with the VWF promoter in both cell types (Fig. 1C, lanes 3 and 4) and demonstrated that the VWF promoter is similarly associated with acetylated histone H3 in both HUVEC and HEK293 cells (Fig. 1C, lanes 7 and 8). However, acetylated histone H4 was shown to be specifically associated with the VWF promoter in HUVEC (Fig. 1C, lane 5), thus demonstrating that the state of acetylation of histone H4 is correlated to the VWF promoter activity.

Inhibition of the VWF Promoter Activity through NFY Is Correlated to Histone H4 Hypoacetylation of the Promoter—It was demonstrated previously that a fragment of the VWF promoter spanning the sequences −90 to +155 functions as a core promoter and is activated in both endothelial and non-endothelial cells, while a fragment that spans the sequences...
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Fig. 2. Interaction of NFY with the repressor element in the VWF promoter decreases association of acetylated histone H4 with the VWF promoter sequences in HEK293 cells. A, schematic representations of the VWF-HGH plasmids containing the wild type and mutant VWF promoter fragments are shown on the left. Solid triangle, labeled NFY represents the mutation in the repressor NFY-binding site. HEK293 cells were stably transfected with VWF-HGH plasmids, and analyses of growth hormone expression were performed as described previously (27). Bar graph represents percent activity of each promoter fragment as compared with that of HGH-1. B, chromatin from HEK293 cells stably transfected with HGH-1, HGH-1K, and HGH-1KY (described in A) were immunoprecipitated with anti-acetylated histone H3 (AH3) and AH4 antibodies. Immunoprecipitated chromatins were subjected to PCR analyses using the actin-specific primers described in Fig. 1B and VWF-specific primers that amplify VWF gene sequences −30 to +155 (described under "Materials and Methods"). Amplified DNA fragments were analyzed on 1.5% agarose gel. C, HEK293 cells stably transfected with HGH-1, HGH-1K, and HGH-1KY (described in A) were exposed to 100 ng/ml TSA, and the levels of growth hormone prior to and 48 h after exposure to TSA were determined. The level of growth hormone expression from each plasmid prior to TSA treatment was considered as 100% (shown by hatched bar), and the level of expression post-TSA treatment (shown by solid bar) was determined as percentage of that obtained prior to TSA treatment. Results are averages of six independent experimental points for each plasmid.

−90 to +247 functions as an endothelial specific promoter, and its activity is repressed in non-endothelial cells (26, 27). Mutation and transfection analysis in HEK293 cells demonstrated that the inhibition of this promoter function in non-endothelial cells is mediated through NFY binding to the novel binding site located at the sequences +226 to +234 (27). To determine whether repression/activation of this promoter fragment in transfected cells is correlated to changes in acetylation status of associated histones, we performed ChIP analysis of the stably transfected HEK293 cells.

The cells were stably transfected with plasmids HGH-1, HGH-1K, and HGH-1KY that contain human growth hormone gene fused to various VWF promoter fragments. Plasmid HGH-1 contains the core VWF promoter fragment (the sequences −90 to +155) that is active in all cell types including HEK293 cells. Plasmid HGH-1K contains wild type sequences −90 to +247 of the VWF promoter that has significantly reduced activity (compared with HGH-1) in HEK293 cells. Plasmid HGH-1KY contains sequences −90 to +247 with 3-base substitution mutations at the repressor-NFY-binding site. We have reported previously that this mutation inhibits NFY interaction with the VWF DNA sequences and results in promoter activation in HEK293 cells to a similar level as that of the core promoter (HGH-1). The levels of growth hormone expression (that is an indicator of these VWF promoters activities) from stably transfected HEK293 cells were reported previously (27) and confirmed by repeating the growth hormone assay from the transfected cells as shown in Fig. 2A.

To determine correlation of these VWF promoter activities to the states of histone acetylation, ChIP analyses were performed using anti-acetylated histone H3 and H4 antibodies and the VWF-specific primers (amplifying a 185-bp fragment corresponding to sequences −30 to +155) for PCR detection as described above for Fig. 1C. Primers that were used in PCR analysis detect endogenous VWF as well as transfected VWF-HGH sequences. However, we do not expect any changes in the levels of acetylated histones that are associated with endogenous VWF in these cells. Thus any differences observed reflect differences in the levels of acetylated histones that are associated with transfected VWF promoter fragments. As control we also used actin-specific primers (as described for Fig. 1B) to demonstrate equal association of actin promoter fragment with acetylated H3 and H4 histones in all transfected cells.

The results demonstrated that there were no significant differences in the levels of acetylated histone H3 that were associated with the VWF promoter sequences among all three transfected cell lines (Fig. 2B, lanes 1–3). However, acetylated histone H4 levels that were associated with the VWF promoters significantly differed in these cell lines (Fig. 2B, lanes 4–6). The promoters that were active, HGH-1 and HGH-1KY, had...
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Fig. 3. HDACs are specifically associated with NFY and the VWF promoter in HEK293 cells. A, chromatin from HUVEC and HEK293 cells was immunoprecipitated with no antibody (Ab), anti-HDAC1, and anti-HDAC2 antibodies. The input panel serves as positive control and represents the total chromatin used as template that was not subjected to immunoprecipitation. The ChIP and input chromatin were used as templates for PCR to detect the VWF promoter sequences. Primers amplified the sequences +155 to +247 of the VWF gene (shown in Fig. 1A). The lane labeled—represents sham-immunoprecipitated chromatin (no antibody). The amplified PCR fragments were analyzed on 1.5% agarose gel. B, nuclear extracts (30 µg) prepared from HUVEC and HEK293 cells were subjected to Western blot analysis and hybridized to anti-HDAC1 and anti-HDAC2-specific antibodies. C, nuclear extracts (50 µg) prepared from HUVEC and HEK293 cells were immunoprecipitated with IgG, anti-NFY-A, and anti-HDAC1 antibodies (represented as IP-Ab). The immunoprecipitated complexes were analyzed by Western blot analysis and hybridized to anti-NFY-A antibody (represented as W-Ab). D, chromatin from HEK293 cells stably transfected with HGH-1K and HGH-1KY were immunoprecipitated with no antibody, anti-HDAC1, and anti-HDAC2 antibodies. The input panel serves as positive control and represents the total chromatin used as template that was not subjected to immunoprecipitation. The ChIP and input chromatin were used as templates for PCR to detect VWF-HGH sequences specifically in the transgene. The primers corresponded to the +153 to +183 region of the VWF gene and the +1 to +30 region of the human growth hormone gene.

The results demonstrated that there was an ~50% increase in the activity of the VWF promoter fragment that contained the intact novel NFY-binding site (Fig. 2C, HGH-1K), whereas TSA treatment had no effect on the activity of the VWF promoter fragments with deletion or mutation of novel NFY-binding sequences (Fig. 2C, HGH-1 and HGH-1KY).

These data demonstrated that the VWF promoter fragments with deletion or mutation of the novel NFY-binding sites (HGH-1 and HGH-1KY) were active in HEK293 cells and had increased association with acetylated histone H4 compared with the VWF promoter fragment that had an intact NFY-binding site and reduced activity (HGH-1K).

Because specific mutation of NFY-binding site in the HGH-1KY plasmid was correlated to an increased association of acetylated histone H4 with the VWF promoter fragment, we hypothesized that NFY may recruit histone deacetylases to the VWF promoter through interaction with the repressor element (the novel NFY-binding site located at the sequences +226 to +234), thus reducing the level of acetylation of histone H4 associated with the VWF promoter and leading to promoter inactivation. Thus, mutation of the NFY-binding site that inhibits NFY interaction with the VWF promoter would be expected to inhibit recruitment of histone deacetylases to the promoter. This results in increased level of histone H4 acetylation, consequently leading to promoter activation.

To test this hypothesis, we first performed experiments in which the effects of histone deacetylase inhibitor trichostatin A on the level of the activities of these promoter fragments were determined. HEK293 cells stably transfected with HGH-1, HGH-1K, and HGH-1KY were untreated or treated with TSA (100 ng/ml), and the growth hormone expressed was determined prior to and 48 h post-treatment. The level of growth hormone in cells expressing each plasmid was considered as 100% prior to treatment.

The results demonstrated that there was an ~50% increase in the activity of the VWF promoter fragment that contained the intact novel NFY-binding site (Fig. 2C, HGH-1K), whereas TSA treatment had no effect on the activity of the VWF promoter fragments with deletion or mutation of novel NFY-binding sequences (Fig. 2C, HGH-1 and HGH-1KY).

These data demonstrated that the VWF promoter fragments with deletion or mutation of novel NFY-binding sites (HGH-1 and HGH-1KY) were active in HEK293 cells and had increased association with acetylated histone H4 compared with the VWF promoter fragment that had an intact NFY-binding site and reduced activity (HGH-1K).
strate that the lack of association of HDACs with VWF promoter in HUVEC is not because of the absence of HDAC proteins in these cells.

To investigate the role of NFY in recruitment of histone deacetylases, we performed immunoprecipitation/Western blot analysis to determine interaction of NFY and histone deacetylases. For these analyses we used anti-HDAC1, anti-NFY-A (as positive control), and anti-IgG (as negative control) antibodies to immunoprecipitate the corresponding proteins from HEK293 and HUVEC cells and subjected the immunoprecipitated protein complexes to Western blot analysis using anti-NFY antibody. The results demonstrated that NFY and HDAC1 are associated in both cell types; however, the level of association is significantly higher in HEK293 cells than in HUVEC (Fig. 3C, lanes 2 and 6). There were no associations of immunoprecipitated IgGs with NFY in either cell type (Fig. 3C, lanes 1 and 4), whereas immunoprecipitated NFY was detected with anti-NFY-A antibody in both cell types (Fig. 3C, lanes 2 and 5), thus demonstrating the specificity of the NFY-A antibody in Western blot analysis.

This result demonstrates that NFY interacts with histone deacetylases, and this association is specifically reduced in endothelial cells.

To demonstrate directly that HDACs are recruited to the VWF promoter through interaction with NFY, we performed ChiP analysis in HEK293 cells stably transfected with plasmids HGH-1K and HGH-1KY. For these analyses chromatin from stably transfected cells were immunoprecipitated with anti-HDAC1 and anti-HDAC2 antibodies as described for Fig. 3A; however, the primers that were used for PCR analysis corresponded to the VWF promoter sequences +153 to +183 and the human growth hormone sequences +1 to +30. By using these sets of primers, a 150-bp fragment corresponding to the sequences spanning the chimeric VWF and human growth hormone gene sequences in the HGH-1K and HGH-1KY transgenes (and not the endogenous VWF promoter sequences) were amplified, thus avoiding ambiguity in interpretation of the data as to whether the results represent the association of HDAC with endogenous VWF or the transgene sequences. The results demonstrated that HDAC1 and HDAC2 are associated with the VWF promoter sequences in the HGH-1K plasmid that contains the wild type NFY-binding site (Fig. 3D, lanes 2 and 3) but not with VWF sequences in the HGH-1KY plasmid that contains the base substitution mutations in the NFY-binding site (Fig. 3D, lanes 5 and 6). The VWF and HGH-specific primers amplified the appropriate 150-bp fragments from all non-immunoprecipitated input chromatin used as control (Fig. 3D, lower panel). These data demonstrate that HDACs are associated with the VWF promoter and that mutation of the NFY-binding site inhibits HDAC association with the VWF promoter, thus demonstrating that NFY binding to the novel repressor element is necessary for the recruitment of the HDACs to the VWF promoter.

Based on these results, we hypothesize that NFY can function as a repressor by recruiting histone deacetylases and that there may be a cell type-specific mechanism of regulating NFY-HDAC1 association.

**Transcription Factor GATA6 Interacts with the VWF Promoter and Associates with NFY in a Cell Type-Specific Manner**—Our previous analysis of the VWF promoter demonstrated that a GATA transcription factor interacts with the VWF promoter sequences, and this interaction was necessary for the promoter activation in endothelial cells (26). Because the binding site for the GATA factor is adjacent to the novel NFY-binding site, we hypothesized that the GATA factor is involved in regulation of the VWF promoter through a cell type-specific mechanism that may involve interaction of GATA and NFY.

To test this hypothesis, first we needed to determine which member of the family of GATA transcription factors interacts with the VWF promoter sequences. For these analyses we performed supershift gel mobility assays using the VWF sequences +209 to +239 (which were previously shown to bind the GATA factor (26)) as probe and nuclear extracts prepared from HUVEC. Nuclear extracts were preincubated with specific antibodies recognizing human GATA2, GATA4, and GATA6 prior to addition of the probe. We excluded GATA1 and GATA5 antibodies from these studies because previous reports (34, 35) have demonstrated that these factors are not expressed in endothelial cells. GATA3 transcription factor was also reported to mediate T-cell-specific gene expression, thus suggesting that it is an unlikely candidate for the regulation of the VWF gene expression (36). The result of supershift gel mobility assay demonstrated that the only antibody that generated a supershift was anti-GATA6 antibody. There were no differences in the pattern of complex formation among nuclear extracts that were preincubated in the absence of antibodies and those that were preincubated with the antibodies of IgG, GATA2, or GATA4 (Fig. 4A).

GATA2 transcription factor is expressed in endothelial cells and participates in the regulation of a number of genes including VCAM and PECAM that are preferentially expressed in endothelial cells (3, 5). However, supershift gel mobility assay demonstrated that GATA2 does not interact with VWF promoter sequences, whereas GATA6 appears to bind to the GATA site. To determine the presence of GATA2 and GATA6 factors in HUVEC, we performed Western blot analysis of the nuclear proteins. The results demonstrated that while both GATA2 and GATA6 are expressed in HUVEC, the level of GATA6 transcription factor appears to be significantly higher in these cells (Fig. 4B). However, this may be due to the efficiencies of the antibodies used to detect GATA2 and GATA6. To demonstrate directly whether GATA6 and/or GATA2 interact with VWF promoter sequences in vivo, we performed ChiP assay using anti-GATA6 and anti-GATA2 antibodies and chromatin prepared from HUVEC. Immunoprecipitated chromatin was subjected to PCR analysis using the VWF-specific primers (amplifying the 92-bp fragment corresponding to VWF sequences +153 to +247) as described above for Fig. 1C. As control we used VCAM-specific primers that amplify a 160-bp region of the VCAM promoter (corresponding to the sequences ~320 to ~161) containing two GATA-binding sites (37), which were reported previously (38) to bind GATA2. VCAM promoter was also shown to bind GATA6 in response to tumor necrosis factor-α stimulation (5). The results of ChiP analysis demonstrated that GATA6 but not GATA2 interacts with endogenous VWF promoter sequences in HUVEC (Fig. 4C, panel VWF, lanes 2 and 3), whereas both GATA2 and GATA6 interact with the VCAM promoter sequences (Fig. 4C, panel VCAM, lanes 2 and 3).

These data confirm the results of gel mobility experiments and demonstrate that GATA6 specifically interacts with the VWF promoter sequences in HUVEC.

To demonstrate whether GATA6 interaction with the VWF promoter sequences is specific to endothelial cells, we performed ChiP assay using anti-GATA6 antibody and chromatin prepared from HUVEC and HEK293 cells. Immunoprecipitated chromatin was subjected to PCR analysis using the VWF-specific primers (amplifying the 92-bp fragment corresponding to the VWF sequences +153 to +247) as described above. The results demonstrated that GATA6 interacts with the endogenous VWF promoter sequences both in HUVEC and HEK293 cells (Fig. 4D). These analyses demonstrated that there was no
preferential binding of GATA6 to the VWF promoter in endothelial cells compared with non-endothelial cells. These results suggest that although GATA6 binding is necessary for VWF promoter activation, it is not sufficient to determine differential regulation of VWF promoter activity in endothelial and non-endothelial cells. However, this does not exclude the possibility that there may be differential interaction of NFY-HDAC and the adjacent GATA6 factor in endothelial and non-endothelial cells that may contribute to the mechanism of differential regulation of the VWF promoter.

To test this hypothesis, we performed immunoprecipitation/Western blot analysis to determine the pattern of interaction of NFY and GATA6 in these two cell types. Mouse IgG, anti-NFY-A, and anti-GATA6 antibodies were used to immunoprecipitate the corresponding proteins, and the complexes were subjected to Western blot analysis using anti-NFY-A antibody. The results demonstrated that GATA6 is specifically associated with NFY in HEK293 cells but not in HUVEC (Fig. 4E).

Although these results do not determine whether GATA6 and NFY directly interact with each other, they demonstrate an association of these factors that is cell type-specific. Based on these results, we hypothesize that the activating function of GATA6 may be modulated through its interaction with other factors including NFY and potentially HDACs that are also associated with NFY. Such interactions may modify or inhibit activating potential of GATA6 in non-endothelial cells without interfering with its DNA binding properties. In endothelial cells, GATA6 may be specifically modified so that it does not interact with NFY and/or it may interact with different cohorts of activators/coactivators to maintain and enhance its transcriptional activating properties.

**DISCUSSION**

Regulation of the VWF promoter fragments that function in an endothelial specific manner involves a number of cis- and trans-acting factors that positively and negatively affect tran-
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**Fig. 5. Model describing the activation and repression function of NFY-binding site in the VWF promoter.** The model represents the VWF region corresponding to sequences +155 to +247. The solid cylinders represent potential nucleosomes, and circles with Ac represent acetylation. In endothelial cells a complex composed of NFY, GATA6, and potentially an endothelial specific HAT (unknown thus represented as HATs?) may be formed. The putative HAT may acetylate the GATA6 and NFY as well as histone H4 in the nucleosomes, and these modifications of trans-acting factors and nucleosomes could facilitate promoter activation. In non-endothelial cells, the absence of endothelial specific HATs could result in the increased pool of NFY associated with HDACs, and a complex consisting of NFY-HDAC-GATA6 may be recruited to the VWF promoters which deacetylate the histone H4 in the nucleosomes and potentially maintain a deacetylated form of GATA6 and NFY. These may contribute to inhibition of the promoter activity by deacetylation of histone H4 and potentially inhibiting the activating function of GATA6 and NFY, thus turning the entire complex to a repressor.

NFY, thus turning the entire complex to a repressor. Contribute to inhibition of the promoter activity by deacetylation of histone H4 and potentially inhibiting the activating function of GATA6 and result in the increased pool of NFY associated with HDACs, and a complex consisting of NFY-HDAC-GATA6 may be recruited to the VWF promoters which deacetylate the histone H4 in the nucleosomes and potentially maintain a deacetylated form of GATA6 and NFY. These may contribute to inhibition of the promoter activity by deacetylation of histone H4 and potentially inhibiting the activating function of GATA6 and NFY, thus turning the entire complex to a repressor.

The effect of histone deacetylase inhibitors on the activity of these promoters. Activation of human MDR1, *Xenopus* HSP70, and transforming growth factor-β type II receptor in response to HDAC inhibitors TSA, sodium butyrate, and MS-275 is mediated by NFY (42–44). HDAC inhibitors were shown to initiate interaction of NFY with HATs leading to activation of transforming growth factor-β type II receptor promoter in human breast cancer cell lines, whereas basal association of NFY and HATs were reported in other systems (44).

We have also demonstrated that the interaction between NFY and the CCAAT sequence is required for the VWF promoter up-regulation in response to irradiation and that NFY and PCAF association in endothelial cells is increased in response to irradiation (30).

Because co-activators with histone acetylating function may contribute to trans-activating function of NFY, we hypothesized that cofactors with histone deacetylating function may contribute to NFY function as repressor. Based on this hypothesis, VWF promoter activity is expected to correlate to the level of acetylation of the histones that are associated with the VWF promoter sequences. In addition, inhibition of NFY binding to the repressor element is expected to result in changes in acetylation pattern of histones associated with the VWF promoter.

Our analyses demonstrated that the endogenous VWF promoter sequences in endothelial cells are specifically associated with acetylated histone H4. Such association was not observed in HEK293 cells that do not express the VWF gene. Furthermore, transfection studies in HEK293 cells demonstrated that the core VWF promoter fragment (sequences −90 to +155, which does not contain repressor elements and is active in HEK293 cells) is also associated with acetylated histone H4, whereas endothelial specific promoter fragment (sequences −90 to +247, that contains the repressor NFY-binding site and is not active in HEK293 cells) has significantly decreased association with acetylated histone H4. Direct evidence that NFY binding to the repressor element mediates the decreased association of acetylated H4 with VWF promoter was obtained when the endothelial specific promoter fragment with mutation in the repressor NFY-binding site was shown to have similar levels of association with acetylated H4 and similar
levels of activity as that of the VWF core promoter fragment. In
addition, histone deacetylase inhibitor TSA was shown to in-
crease the activity of the transfected VWF promoter fragment
that contains an intact repressor NFY-binding site but not that
of mutant or core promoter fragment. These results are con-
sistent with the hypothesis that NFY factor recruits histone
deacetylases to the VWF promoter when functioning as re-
pressor. Further evidence to support this hypothesis was obtained
by IP/Western blot analysis that demonstrated the association
of NFY and HDAC1 specifically in HEK293 cells, and also ChIP
analysis demonstrating that HDACs are specifically recruited
to the endogenous VWF promoter sequences in HEK293 cells
but not in HUVEC. Furthermore, HDACs were shown to be
associated with VWF-HGH transgene (HGH-1K) containing
the wild type but not that containing the mutated NFY-binding
site (HGH-1KY) in transfected HEK293 cells.

These data not only support the hypothesis that the function
of NFY as a repressor is mediated through recruitment of
HDACs, they also demonstrate that there is cell type specificity
to the pattern of NFY association with HDACs.

Based on these results, we also hypothesize that there are
two pools of NFY-containing complexes in cells, one that is
associated with HATs and one that is associated with HDACs.
This hypothesis is consistent with previous observations that
HDAC inhibitors increase NFY-dependent promoter activation
as discussed above. Based on this hypothesis, HDAC inhibitors
may release NFY from HDAC complexes and thus increase the
level of NFY that can associate with HATs. There are observa-
tions that in some cell types there are basal NFY-HAT com-
plexes (42) while in others this complex is observed only in
response to inducers or HDAC inhibitors (44), and there is cell
type specificity to the pattern of NFY-HDAC and potentially
NFY-HAT complex formations. Recently, the dual function of
NFY as both activator and repressor of transcription of SHP-1
gene in MCF7 cells was reported (40). Although in this report
two distinct binding sites for NFY were also involved, both
binding sequences consisted of inverted CCAAT sequence. The
binding of NFY to the distal CCAAT element was shown to
enhance transcription, whereas binding to the proximal site
was reported to repress the promoter activity. Furthermore,
the binding of the proximal site eliminated the enhancing
effect of TSA on the promoter activity (40). This further sup-
ports our hypothesis that NFY can interact with complexes
that contain HDACs and function as repressor.

Other ubiquitously expressed transcription factors such as
YY1 and NF-κB are also shown to function as activators and
repressors based on their interaction with HATs and HDACs
(23, 24). Specifically, the binding sequence for YY1 is im-
planted in the role of this factor as activator or repressor (25).
Thus, NFY provides another example of a ubiquitously ex-
pressed trans-acting factor whose function can be modulated
based on its interaction with specific type of coactivators.
The cell type-specific pattern of association of NFY with HDACs
also provides a mechanism for this ubiquitously expressed fac-
tor to participate in cell type-specific regulation of gene
expression.

Interaction of NFY with other DNA binding transcription
factors is also reported to participate in the regulatory function
of NFY in mediating promoter activation (41). We have re-
ported previously (26) the presence of a GATA-binding se-
quence in the VWF promoter that is necessary for promoter
activation in endothelial cells. This GATA element is situated
directly adjacent to the repressor NFY-binding sequence, thus
raising the possibility that the interaction of NFY and the
GATA factor that binds to the GATA site may be involved in
cell type-specific regulation of the VWF promoter activity (27).

To test this hypothesis we first identified GATA6 as the mem-
ber of the GATA transcription factor family that interacts with
the GATA site in the VWF promoter, and we then determined
the pattern of GATA6 and NFY interaction in endothelial
and non-endothelial cells. Our results demonstrated that GATA6
is specifically associated with NFY in HEK293 cells but not in
HUVEC. Because these results were obtained by IP/Western blot
analysis, they do not demonstrate whether this association
is direct or indirect; however, the cell type specificity of the
association suggests a potential mechanism for participation of
these factors in regulating the endothelial cell-specific activa-
tion of the VWF promoter. Based on these data we propose a
hypothesis (Fig. 5). The complexes including NFY-HDAC and
GATA6 are formed in non-endothelial cells, which may result
in deacetylation of GATA6 as well as recruiting HDACs to
the VWF promoter. This complex may inhibit the transcriptional
activity of GATA6 as well as deacetylation of the histones on
the VWF promoter, thus rendering the promoter inactive in
non-endothelial cells. The lack of this complex formation in
endothelial cells may result in the presence of a pool of GATA6
transcription factors that are not deacetylated and, thus, can
function as an activator of the VWF promoter. This hypothesis
does not exclude the possibility that endothelial cell-specific
transcription factors or coactivators may interact with GATA6
to prevent association of GATA6 with NFY-HDAC complexes or
recruit HATs specifically to GATA6 and/or disrupt NFY-HDAC
associations specifically in endothelial cells. Regardless of the
potential mechanism, the presence of NFY in a complex that
contains GATA6 and HDAC specifically in non-endothelial
cells provides a potential mechanism for cell type-specific reg-
ulation of the VWF promoter by these transcription factors that
does not depend on their cellular expression pattern. However,
potentially cell type-specific post-translational modifications
of these factors may lead to these different associations and com-
plex formations, thus leading to their differential regulation of
the VWF promoter in endothelial and non-endothelial cells.

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J. Biol. Chem. 2003, 278:8385-8394.
doi: 10.1074/jbc.M213156200 originally published online January 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213156200

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