YY1 Binds Five cis-Elements and Trans-activates the Myeloid Cell-restricted gp91phox Promoter*

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Four transcriptional activating cis-elements within the gp91phox promoter bind a protein complex of similar mobility and binding specificity, denoted BID (binding increased during differentiation). The intensity of BID complexes increases upon myeloid cell differentiation, coincident with induction of gp91phox expression, and BID competes with the transcriptional repressor CDP for binding to each of these promoter elements. To determine the identity of BID, an expression library was ligand screened with the BID-binding site that surrounds the −145-base pair (bp) region of the gp91phox promoter. One recovered factor that exhibits the expected binding specificity is YY1, a ubiquitous multifunctional transcription factor. BID complexes that form with the four binding sites within the gp91phox promoter are disrupted by YY1 antiserum, and a fifth YY1-binding site was detected in the −412-bp promoter region. Overexpression of YY1 in transient co-transfection assays trans-activates a minimal promoter containing two copies of the −145-bp binding site from the gp91phox promoter. Neither the level of YY1 protein nor DNA binding activity increases during myeloid cell differentiation. These studies identify a target gene of YY1 function in mature myeloid cells, and demonstrate that YY1 function can be controlled during myeloid development by the modulation of a competing DNA-binding factor.

Phagocytic blood cells such as monocyte/macrophages and neutrophils use the NADPH oxidase to produce a respiratory burst to kill microbes. Several subunits comprise the NADPH oxidase, including p67phox, p47phox, p22phox, and gp91phox (1). The gp91phox gene is transcriptionally controlled and is expressed nearly exclusively in mature myeloid cells (2). Previously we demonstrated that the −450 to +12 base pair (bp) region of the gp91phox promoter is capable of directing reporter gene expression in a subset of monocyte/macrophages in transgenic mice (3), and responds to interferon (IFN-γ stimulation in transfected myeloid cell lines (4). An enhancer region located 50 kilobases upstream of the proximal promoter is additionally required to direct appropriate expression of gp91phox in the full spectrum of mature myeloid cells (5).

Several DNA-binding proteins interact with the −450 to +12-bp region of the gp91phox promoter (Fig. 1). These include the transcriptional repressor CCAAT displacement protein (CDP) (6–8), as well as several transcriptional activators including the CCAAT-displacement factor CP1 (4, 6, 7), IFN regulatory factor (IRF)1, IRF-2 (8, 9), PU.1 (10–12), IFN consensus sequence-binding protein (11), Elf-1 (12), and an unidentified factor denoted BID (binding increased during differentiation) (8, 9). In immature myeloid cells, the transcriptional repressor CDP binds to at least five sites in the promoter, preventing binding of transcriptional activators to overlapping binding sites, and the gp91phox protein is not expressed. However, CDP DNA binding activity is post-translationally down-regulated in mature myeloid cells (6, 7), allowing transcriptional activators to bind to the promoter and induce gp91phox expression. Previously we reported that BID binds to four sites within the −450 to +12 bp gp91phox promoter (8, 9). These conclusions were based on eletrophoretic mobility shift assay (EMSA) analysis that demonstrated complexes of similar mobility and binding specificity with each of four promoter probes (8, 9). Mutations of the putative BID-binding sites surrounding the −355, −225, and −145 bp regions of the gp91phox promoter result in decreased promoter activity in PLB-855 myeloid cells in response to IFN-γ stimulation (9). Truncation of the gp91phox promoter to −102 to +12 bp removes four CDP-binding sites and reveals a promiscuous promoter that is active in some cells not expressing the endogenous gp91phox gene (7). Specific ablation of a BID-binding site at −90 bp decreases this promiscuous promoter activity by 50% in HEL cells (8). These data indicate that BID functions as a transcriptional activator. Data base searching with the four putative BID-binding sites revealed no common consensus binding sites for known transcription factors. Eklund and Kakar (13) reported the cloning of a novel component of the BID complex (denoted TF1phox in that report). However, data from Yamit-Hezi et al. (14) demonstrate that this clone is a bacterial contaminant present in some commercially available libraries. Hence, the identity of the BID complex remained to be identified.

We undertook a molecular cloning approach to identify BID. Ligand screening of a A511 HeLa cell cDNA expression library was performed using as a probe the −145-bp BID-binding site derived from the gp91phox promoter. One sequence specific DNA-binding factor obtained is YY1, a ubiquitously expressed multifunctional member of the GLI Krüppel-related family of zinc finger transcription factors. Further examination of the −450 to +12 bp gp91phox promoter revealed a fifth potential YY1-binding site. YY1 is present in all five identified BID

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1 The abbreviations used are: bp, base pairs; BID, binding increased during differentiation; CDP, CCAAT displacement protein; EMSA, electrophoretic mobility shift assay; IFN, interferon; FMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; IRF, IFN regulatory factor.
complexes within the gp91<sub>phox</sub> promoter, and transient transfection studies confirm that YY1 functions as a transcriptional activator of the gp91<sub>phox</sub> promoter.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—**A β-globin TATA box minimal promoter fragment was excised from a human growth hormone gene reporter vector (gift of Ellis Neufeld, Harvard) with HindIII and BamHI and cloned into the luciferase vector, pXPF2 (15), that was digested with HindIII and BglII. This construct was digested with BamHI, de-phosphorylated with shrimp alkaline phosphatase (Amersham Pharmacia Biotech/U. S. Biochemical, Cleveland, OH), and ligated to phosphorylated −145 Core or −145 mut oligonucleotides (see below). The nucleotide sequence was determined for constructs containing a dimer of each oligonucleotide, and those with a dimer in the forward orientation were prepared by cesium chloride ultracentrifugation to be used in transient transfections (see below).

**Cell Culture and Transient Transfection Assays—**HeLa human cervical choriocarcinoma cells, K562 human chronic myelogenous leukemia cells, and HEL human erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, MD). The PLB-985 human myelomonoblastic cell line (16) was a gift of Thomas Rado (Birmingham, AL). Suspension cells (HEL, K562, and PLB-985) were grown in RPMI 1640 medium with 10% fetal bovine serum or Fetal Clone III (Bovine Serum Product, HyClone, Logan, UT) and 0.2 mM glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin at 37 °C and 5% CO2. Adherent cells (HeLa) were grown in similarly supplemented Dulbecco’s modified Eagle’s media.

Cells were diluted in media to a concentration of 10⁷ cells/300 μl and placed into electroporation cuvettes (0.4-cm gap). One microgram of luciferase test plasmid and 5 μg of CMV driven expression plasmid or CB6−YY1 (17) (gifts of Kenneth Walsh, Tufts) were added to each cuvette. Each sample also contained 0.25 μg of cytomegalovirus promoter/β-galactosidase (CMV/β-gal) plasmid that serves as an internal control for transfection efficiency. Samples were electroporated using a Bio-Rad Gene Pulser at 960 microfarads and 220 V, re-suspended in 10 ml of media and incubated at 37 °C for 24 h. Cells were harvested with PBS, resuspended in 100 μl of 1% lysis buffer (Promega Inc., Madison, WI), and incubated at room temperature for 15 min. Twenty microliters of cell lysate was assayed for luciferase activity as described by the manufacturer (Promega Inc.) using a Bio-Rad TD-20/20 luminometer (Promega, Inc., Madison, WI). Isolated DNA was digested with restriction enzymes prior to Southern blotting for cloning into Bluescript (Stratagene, La Jolla, CA). The nucleotide sequence was determined for constructs containing a dimer of each oligonucleotide, and those with a dimer in the forward orientation were prepared by cesium chloride ultracentrifugation to be used in transient transfections (see below).

**In Vitro DNA Binding Protein Assays—**Cell nuclear extracts were prepared by the method of Dignam et al. (19). PLB-985 cells were treated with agents that induce gp91<sub>phox</sub> expression as described previously (9). To prepare fractionated extract, six liters of K562 cells were grown to log phase and nuclear extract was prepared as described above. Nuclear extract was then fractionated by heparin-agarose (Sigma) chromatography using a stepwise gradient of KCl in Dignam Buffer D supplemented with protease inhibitors. Fractions exhibiting abundant BID binding activity (0.2–0.3 μM KCl) were pooled and dialyzed to 0.1 μM KCl. For mini-nuclear extracts, 10⁶ HEL cells were transiently transfected with 20 μg of CB6− or CB6-YY1 expression plasmids as described above, incubated for 12 h, and mini-nuclear extracts prepared as described (20).

Complimentary oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP. Radiolabeled probes were purified as described previously (8). EMSA was performed as described previously (6), using 4–9 μg of nuclear extract and 0.1–0.5 μg of poly(dI-dC). Non-radioactive competitor oligonucleotides were added to designated samples and incubated on ice for 12 min. One to two micrograms of YY1 antisense (catalog number sc-1703x, Santa Cruz Biotech, Santa Cruz, CA) or Ets-2 antisense (catalog number sc-351x, Santa Cruz Biotech) was added to designated samples and incubated on ice for 40 min. Radiolabeled oligonucleotide probe (20,000 cpm) was added (except where otherwise indicated) and samples were incubated on ice for an additional 30 min. The reactions were loaded onto native 6% polyacrylamide gels (except where otherwise indicated) and electrophoresis was performed at 25 mA for 1.25 h at 4 °C in 0.5 × TBE. Gels were then dried and exposed to x-ray film at −70 °C.

**Oligonucleotides Used in EMSA—**Complimentary oligonucleotides were synthesized on an Applied Biosystems model 394 synthesizer. Sequences correspond to the upper strand of the human gp91<sub>phox</sub> promoter (6). Mutated bases are underlined, and all oligonucleotides contain BamHI linkers (not shown): −90 (−102 to −65 bp), 5′-ggctgaaaaggaaagacgctvecgeggagttccctc-3′; −145 Core (−155 to −130 bp), 5′-gaagttttcagagttcctgcttcct-3′; −145 mut (−155 to −125 bp), 5′-aaagttttaatcgaactacagtttctgcttcct-3′; −225 Core (−240 to −215 bp), 5′-aaagtgtttaaaatcgaactacagtttctgcttcct-3′; −355 Core (−369 to −344 bp), 5′-aatctaacacgagttcctgcttcct-3′; −412 (−424 to −399 bp), 5′-gaagtttaatc gaactacagtttctgcttcct-3′; −422 (−434 to −403 bp), 5′-aatctaacacgagttcctgcttcct-3′; −70 °C. A total of 2.5 × 10⁶ cpm of [γ-32P]dCTP using random priming. Probe was added to 10⁴ cpm/ml of 1 × binding buffer (25 μM HEPES (pH 7.5), 1 μM dithiothreitol, 10% glycerol, 50 μM NaCl, 0.05% lauryldimethylamine oxide (Calbiochem), 1 mM EDTA). Filters were rinsed briefly in TNE-50 (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol).

**Ligand Screening a ggt11 cDNA Expression Library—**A ggt11 cDNA expression library derived from HeLa cells (CLONTECH, Palo Alto, CA) was a generous gift of Dr. Saw Yin Oh (Indianapolis, IN). Y1090 cells were incubated with bacteriophage for 15 min at 37 °C and plated at a density of 50,000 plaques/150-mm plate, incubated at 42 °C for 5 h, and plaque assays. Phage eluted from plaques was added to 10⁶ HEL cells per plate. Each phage was rescreened with 100 μl of 1% lysis buffer (Promega Inc., Madison, WI), and incubated at room temperature for 15 min. Twenty microliters of cell lysate was assayed for luciferase activity as described by the manufacturer (Promega, Inc.) using a Lumat LB 9501 (Berthold, Gaithersburg, MD) luminometer. β-Gal activity was detected as described (18) and used to adjust luciferase values to correct for differences in transfection efficiency. Each sample was transfected in duplicate, and independent plasmid preparations of each construct were used in five independent experiments.
sequence of subcloned fragments was determined by the dideoxy chain termination method using M13 and T3 primers. Obtained sequences were used to search the GenBank data base.

Western Analysis—Nuclear extracts were quantitated using the method of Bradford (23). SDS loading dye was added to 40 mg of nuclear extract and the samples were boiled for 10 min then loaded onto a 4–20% Tris glycine gel (Novex, San Diego, CA) and electrophoresis was performed at 180 V for 1.5 h at 4 °C. The proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) for 2 h at 80 V, and the membrane was blocked in PBS + 0.1% Tween 20 (PBS-T) containing 5% low fat milk. Following blocking, the membranes were washed three times with PBS-T and incubated with an antiserum raised against the full-length YY1 protein (Santa Cruz Biotech., Inc) diluted 1:20,000 in blocking buffer for 1 h at room temperature. The membrane was washed five times in PBS-T, and chemiluminescent detection was performed according to the manufacturer’s instructions (Amerham Pharmacia Biotech).

FIG. 2. BID is ubiquitously expressed and is not induced during phagocyte maturation. A, BID is not a myeloid specific complex. EMSA analysis using the −145 Core oligonucleotide as probe was performed as described under “Experimental Procedures” using nuclear extracts derived from PLB-985, HeLa, K562, or HEL cells. Competitor −145 Core or −145 mut oligonucleotides (60 ng) were added where indicated. Arrows denote complexes that exhibit the binding specificity of BID. B, BID binding is not increased by agents that induce gp91phox expression. EMSA analysis was performed as described under “Experimental Procedures” using 4 µg of nuclear extract derived from either PLB-985 cells, or PLB-985 cells treated with PMA or IFN-γ. The −145 Core BID-binding site was used as a probe. The oligonucleotides −145 Core or −145 mut (60 ng) were added as competitors where indicated. Relative intensities of nonspecific EMSA complexes serve as internal controls for protein loading and integrity of the nuclear extracts. An arrow indicates the position of the BID complex.

FIG. 3. Isolation of a sequence-specific DNA-binding clone. A Agt11 HeLa expression library was screened as described under “Experimental Procedures.” The upper half of each filter was probed with a concatemer of the −145 Core-binding site, while the lower half of each filter was probed with the −145 mut concatemer that no longer binds the BID protein. A representative clone exhibiting sequence-specific DNA binding activity is shown on the right, and a nonspecific clone is presented on the left.

sequence of subcloned fragments was determined by the dideoxy chain termination method using M13 and T3 primers. Obtained sequences were used to search the GenBank data base.

Western Analysis—Nuclear extracts were quantitated using the method of Bradford (23). SDS loading dye was added to 40 µg of nuclear extract and the samples were boiled for 10 min then loaded onto a 4–20% Tris glycine gel (Novex, San Diego, CA) and electrophoresis was performed at 180 V for 1.5 h at 4 °C. The proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) for 2 h at 80 V, and the membrane was blocked in PBS + 0.1% Tween 20 (PBS-T) containing 5% low fat milk. Following blocking, the membranes were washed three times with PBS-T and incubated with an antiserum raised against the full-length YY1 protein (Santa Cruz Biotech., Inc) diluted 1:20,000 in blocking buffer for 1 h at 25 °C. The membrane was washed three times with PBS-T and secondary antibody conjugated to horseradish peroxidase was diluted 1:20,000 in PBS-T containing 5% low fat milk and incubated with the membrane for 1 h at room temperature. The membrane was washed five times in PBS-T, and chemiluminescent detection was performed according to the manufacturer’s instructions (Amerham Pharmacia Biotech).

RESULTS

The BID DNA Binding Activity Is Not Induced during Myeloid Cell Differentiation—Numerous DNA-binding proteins interact with the proximal gp91phox promoter to direct myeloid cell-restricted expression (Fig. 1). The transcriptional repressor CDP binds to multiple sites in the proximal promoter in undifferentiated myeloid cells, thus excluding the binding of transcriptional activators to overlapping binding sites. Myeloid precursor cells treated with agents that induce gp91phox expression, such as phorbol ester (PMA), dimethylformamide, or IFN-γ exhibit decreased CDP DNA binding activity and a concomitant increase in the intensity of BID complexes (6, 7, 24, 25). CDP and BID bind to the gp91phox promoter in a mutually exclusive manner (7). To facilitate study of BID, a truncated oligonucleotide was created which lacks several of the bases necessary for CDP binding to the −145 bp region of the gp91phox promoter. This oligonucleotide (−145 Core) was
FIG. 4. The BID complex that binds the −145 Core site contains YY1. A, YY1 binds the −145 Core site. EMSA analysis was performed as described under "Experimental Procedures" using the −145 Core oligonucleotide as probe and nuclear extract derived from HEL cells. The −145 Core or −145 mut competitor oligonucleotides (60 ng) were added where indicated. YY1 (αYY1) or Ets-2 (αEts2) antiserum (1–2 μl) was added where indicated. An arrow denotes the position of the BID complex. B, complexes of similar mobility and binding specificity form with the −145 Core and YY1 consensus binding sites. EMSA analysis was performed as described under "Experimental Procedures" using −145 Core or YY1 consensus binding site probes and nuclear extract derived from HEL cells. The −145 Core, −145 mut, YY1, or YY1 mut oligonucleotides (60 ng) were added as competitors where indicated. An arrow denotes the position of the BID (YY1) complex. C, overexpression of YY1 leads to an increase in the intensity of the BID complex. EMSA analysis using mini-nuclear extract and the −145 Core probe was performed as described under "Experimental Procedures." Five micrograms of mini-nuclear extracts derived from HEL cells transfected with the CB6− parent vector or the CB6-YY1 expression vector were added where indicated. The −145 Core or −145 mut oligonucleotide competitor (60 ng) or YY1 or Ets-2 antiserum (1–2 μl) was added where indicated. An arrow denotes the BID (YY1) complex.
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YY1 interacts with multiple sites within the gp91phox promoter. A, YY1 binds to each of four BID-binding sites. EMSA analysis was performed as described under “Experimental Procedures.” Each of the four BID-binding sites: −90, −145 Core, −225 Core, and −355 Core was used as probe without (lanes 1, 4, 7, and 10) or with heparin-fractionated nuclear extract derived from K562 cells (all other lanes). YY1 antiserum (1–2 μl) was added where indicated. An arrow denotes the position of the YY1 complex. B, high resolution of YY1 binding to the −225 bp binding site. EMSA was performed as described under “Experimental Procedures” using the −225 Core-binding site probe and heparin-fractionated nuclear extract isolated from K562 cells. To clearly resolve the BID complex, 60 ng of an oligonucleotide competitor containing a mutation which no longer binds the BID complex (BID mut), but retains binding of IRF members (8) was added (lanes 2–4). YY1 or Ets-2 antiserum (1–2 μl) was added where indicated. An arrow denotes the position of the EMSA complex containing YY1.

The −145 Core oligonucleotide probe was used to assess the distribution of the BID binding activity in the absence of competing CDP complexes (Fig. 2A). The BID complex is disrupted upon addition of homologous oligonucleotide competitor (lanes 3, 6, 9, and 12) but not by the −145 mut competitor which no longer binds the BID complex (lanes 4, 7, 10, and 13). The BID complex is present in nuclear extracts derived from PLB-985 (lanes 2–4), HeLa (lanes 5–7), K562 (lanes 8–10), and HEL (lanes 11–13) cells. Thus, expression of BID is not lineage specific. There are also faster migrating complexes in both the HeLa extract (lanes 5–7) and the K562 extract (lanes 8–10) which behave with the same binding specificity as the previously identified slow mobility BID complex. We speculate that these may correspond to products of partial proteolysis of BID.

EMSA was performed using the −145 Core binding site probe and nuclear extracts derived from PLB-985 cells treated with agents that induce gp91phox expression and were previously found to induce increased BID complex intensity (9, 25). The BID complex, which is disrupted with the −145 Core oligonucleotide competitor (Fig. 2B, lanes 3, 6, and 9) but not with the −145 mut oligonucleotide (lanes 4, 7, and 10), is as intense in nuclear extract derived from untreated PLB-985 cells (lanes 2–4) as nuclear extracts derived from PLB-985 cells treated with PMA (lanes 5–7) or IFN-γ (lanes 8–10). Effective induction of the PLB-985 cell cultures was confirmed by demonstrating the down-regulation of CDP DNA binding activity by EMSA analysis (data not shown). Hence, increased intensity of BID complexes with composite BID/CDP probes upon induction of gp91phox expression is the result of decreased binding of CDP to overlapping binding sites.

Cloning of the BID DNA Binding Activity—Searches of the Transcription Factor Sites data base (Genetics Computer Group Inc., Madison, WI) (26) with the −145 Core BID-binding site sequence produced no matches with consensus binding sites for known transcription factors. Hence, a molecular approach was taken to determine the identity of the BID protein. Because BID is ubiquitously expressed (Fig. 2A), ligand screening was performed with an agar11 HeLa cDNA expression library and the concatenated −145 Core BID-binding site probe. The −145 Core site demonstrates the cleanest EMSA complex and the highest affinity for the BID complex. 2 The −145 mut oligonucleotide, which contains a 4-bp mutation and no longer binds the BID complex, was used as a probe to examine the sequence specificity of recovered DNA binding activities.

Approximately 2.5 × 10⁶ plaques were screened, and 43 reproducibly positive DNA-binding clones were examined for binding specificity. Three clones bind to the wild type −145 Core probe, but not to the −145 mut probe (Fig. 3). The other 40 clones encode nonspecific DNA-binding factors as they bind both the −145 Core and −145 mut probes, and were not pursued further. A search of the GenBank data base revealed that the determined nucleotide sequence of a sequence specific clone matches the cDNA sequence of YY1 (data not shown), a ubiquitous multifunctional transcription factor that is a member of the GLI Krüppel zinc finger factor family (27). The recovered

2 B. M. Jacobsen, unpublished observations.
cDNA encompasses amino acid 85 to the stop codon and a portion of the 3' untranslated region, and includes the region of YY1 previously reported to contain the DNA-binding domain (28).

Antiserum directed against YY1 was tested in EMSA analysis using the -145 Core probe (Fig. 4A). The BID complex that forms upon addition of nuclear extract derived from HEL cells (lane 2) is disrupted by homologous oligonucleotide competition (lane 3), but not by the -145 mut oligonucleotide competitor (lane 4), and is also abolished upon addition of YY1 antiserum (lane 5). The other complexes visible in the lanes do not exhibit the appropriate binding site specificity for BID and are not affected by the YY1 antiserum. Also, the BID complex is not affected by an antiserum directed against Ets-2 (lane 6), thus demonstrating the specificity of the disruption by YY1 antiserum.

Additional EMSA studies were performed using a previously described YY1-binding site oligonucleotide to compare the behavior of BID and YY1. The -145 Core site probe (Fig. 4B, lanes 1–6) produces the BID complex (lane 2) which is disrupted by homologous competitor oligonucleotide (lane 3) and not by the -145 mut competitor (lane 4). The BID complex is also disrupted by the YY1 consensus binding site competitor (lane 5), but is not disrupted by a mutated YY1-binding site competitor (lane 6). Additionally, EMSA using the YY1 consensus binding site as a probe (Fig. 4B, lanes 7–10) demonstrates a complex of similar mobility (lane 8) and binding specificity (lanes 9 and 10) as the BID complex formed with the -145 Core probe. EMSA studies with nuclear extract derived from HeLa cells and the -145 Core site probe demonstrate that the previously detected faster migrating complexes (Fig. 2A) are also disrupted by the YY1 specific antibody (data not shown). A complex of slower mobility also binds to the -145 Core site (lane 2). However, this complex does not contain YY1 because it is disrupted by both homologous and mutant competitors (lanes 3 and 4), and is not disrupted by either the YY1 consensus oligonucleotide (lane 5) or YY1 antiserum (Fig. 4A, lane 5).

Nuclear extracts prepared from HEL cells transiently transfected with the CB6-YY1 expression vector (or empty parental expression vector) were analyzed by EMSA using the -145 Core site probe (Fig. 4C). The BID complex is greatly enhanced in cells overexpressing YY1 (lane 3) compared with those transfected with the parental CB6 expression vector (lane 2). The intensified complex is disrupted by competition with homologous oligonucleotide (lane 4) and not disrupted by the -145 mut oligonucleotide competitor (lane 5). The BID (YY1) complex is also supershifted upon addition of YY1 antiserum (lane 6), but is unaffected by antiserum directed against Ets-2 (lane 7). These results provide additional evidence that YY1 is present in the BID complex, and demonstrate that YY1 is either the
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YY1 binds to multiple sites within the gp91<sub>phox</sub> promoter—In previous work to biochemically purify and clone BID, nuclear extract derived from K562 cells was fractionated by heparin chromatography for use in DNA-affinity chromatography (data not shown). This partially purified material exhibits abundant BID activity in EMSA analysis (data not shown). Previous EMSA studies using four sites of the gp91<sub>phox</sub> promoter demonstrated that a complex of similar size and binding specificity forms with each element, suggesting that the same factor binds to each of the sites (8, 9). EMSA analysis using YY1 antiserum and a YY1 consensus oligonucleotide competitor demonstrated that YY1 binds the −145 site (Fig. 4, A and B). To determine if YY1 also binds the other three BID-binding sites, EMSA was performed with heparin-fractionated K562 cell nuclear extract and YY1 antiserum (Fig. 5A). Each of the BID sites derived from the gp91<sub>phox</sub> promoter (−90, −145 Core, −225 Core, and −355 Core) was used as a probe. The BID complex is formed upon addition of fractionated K562 nuclear extract with each of the four probes; −90 (lane 2), −145 Core (lane 5), −225 Core (lane 8), and −355 Core (lane 11). Importantly, the BID complex formed with each of the four probes is ablated upon addition of the YY1 antiserum (lanes 3, 6, 9, and 12).

Both the −90 and −225 bp BID-binding sites within the gp91<sub>phox</sub> promoter also contain IFN-stimulated response element motifs that bind members of the IRF family (8, 9). Because the −225 Core site binds IRF proteins with a high affinity, the IRF complex makes it difficult to detect the BID complex of similar mobility (Fig. 5A, lane 8). Previous studies with the −90-bp element identified a mutation (BID-mut) that specifically ablates the binding site for BID (8). The BID-mut competitor was used to disrupt the IRF complex that forms with the −225 Core probe, clearly revealing the BID complex (Fig. 5B, lane 2). Addition of YY1 antiserum abolishes the BID complex (lane 3), while Ets-2 antiserum does not affect the complex (lane 4). Thus YY1 is a component of the BID complex which forms with four distinct sites within the gp91<sub>phox</sub> promoter.

Alignment of a consensus YY1-binding site (29) with each of the YY1-binding sites within the gp91<sub>phox</sub> promoter reveals strong similarity (Fig. 6A). The −90 bp and −225 bp elements exhibit a 7 of 9 bp match, while the −145 bp and −355 bp sites exhibit a 9 of 9 bp match. Interestingly, the −90 bp and −225 bp sites, which also contain IFN-stimulated response element motifs, do not contain the 5′-ATG-3′ core motif that is invariant in the YY1 consensus binding sequence (29). Further analysis of the −450 to +12 bp gp91<sub>phox</sub> promoter revealed a fifth putative YY1-binding site in the −412-bp region. Alignment of this element with the YY1 consensus site demonstrates an 8 of 9 bp match (Fig. 6A). EMSA using an oligonucleotide to this region as a probe and heparin fractionated nuclear extract derived from K562 cells (Fig. 6B) demonstrates the presence of a complex (lane 2) that is immunoreactive with YY1 antiserum (lane 3), but is not disrupted by Ets-2 antibody (lane 4). This complex also exhibits similar mobility and sequence specificity as the BID complex formed with the other four sites (data not shown).

To our knowledge this is the first report of YY1 regulating a myeloid cell-specific gene. To determine if the level of YY1 protein is regulated during myeloid differentiation, a Western blot was performed using nuclear extracts isolated from differentiated and undifferentiated myeloid cell lines, as well as non-phagocytic cell lines (Fig. 7). Nuclear extracts derived from PLB-985 cells (lane 1), PLB-985 cells treated with PMA and differentiated into macrophages (lane 2), PLB-985 cells treated with IFN-γ (lane 3), PLB-985 cells treated with dimethylformamide and differentiated into granulocytes (lane 4), HeLa cells (lane 5), K562 cells (lane 6), and HEL cells (lane 7) were analyzed. The blot was probed with YY1 antiserum, and one immunoreactive band of approximately 68 kDa is detected in each of the extracts. Although the predicted size of YY1 is 44 kDa, it has previously been reported that YY1 exhibits a mobility of 68 kDa on SDS-polyacrylamide gel electrophoresis (30), most likely because of its highly charged amino terminus. YY1 is present in all cell types examined, consistent with previous reports of the ubiquitous expression of YY1. No reproducible difference was detected in the level of YY1 protein present in cells that are induced to differentiate into mature myeloid cells (lanes 2 and 4) versus those that are not (lane 1). A subtle increase in YY1 is apparent in the extract treated with IFN-γ (lane 3). However, this is likely due to an increased amount of protein loaded, because this extract demonstrates a similar increase in Sp1 levels (data not shown). Western blots performed with a different YY1 antibody and independent preparations of nuclear extracts demonstrated no reproducible differences in YY1 levels in differentiated myeloid cell extracts (data not shown).

Binding of YY1 to the gp91<sub>phox</sub> Promoter Increases in Terminally Differentiated Myeloid Cells—EMSA studies were performed using the −182 to −112 bp region of the gp91<sub>phox</sub> promoter, which contains overlapping binding sites for CDP and BID. Addition of HEL nuclear extract to a limiting amount of probe results in a strong CDP complex (Fig. 8A, lane 1). Addition of an oligonucleotide containing a high affinity CDP-binding site (E36) disrupts binding of CDP to the probe, and a faster migrating complex (BID) becomes apparent (lane 2). Addition of YY1 antiserum disrupts the BID complex (lane 3), confirming the presence of YY1 within the BID complex.
Additional studies were performed to examine whether binding of YY1 to the gp91phox promoter increases during myeloid differentiation. A strong CDP complex (but no YY1 complex) is produced by nuclear extract isolated from immature myeloid cells (Fig. 8B, lane 1). In contrast, nuclear extract isolated from terminally differentiated myeloid cells fails to produce a CDP complex, and a faster migrating complex is detected binding to the probe (lane 2). The faster migrating complex is disrupted upon addition of YY1 antiserum (lane 3). Thus, YY1 binding to the gp91phox promoter is increased in differentiated myeloid cell extract where CDP DNA binding activity is down-regulated.

**YY1 Trans-activates a Minimal Promoter via the −145 Core Element of the gp91phox Promoter**—Our previous studies demonstrated that deletion or mutation of the BID-binding sites in the gp91phox promoter results in decreased promoter activity, suggesting that YY1 is a transcriptional activator (8, 9). Transient co-transfection studies were performed to directly determine if YY1 trans-activates the gp91phox promoter. A dimer of the wild type −145 Core BID-binding site, or a dimer of the −145 mut oligonucleotide, was cloned upstream of the β-globin minimal promoter linked to a luciferase reporter gene. The luciferase constructs were transiently transfected into HEL cells with either the pCB6 parental vector or the pCB6-YY1 expression vector. Luciferase activity expressed by the wild type −145 Core construct is stimulated nearly 10-fold upon overexpression of YY1 (Fig. 9). The −145 mut construct, which lacks the −145-bp YY1-binding site, is stimulated 3-fold, similar to the stimulation observed with the promoterless vector (data not shown). Overexpression of YY1 was confirmed by Western blot analysis of transfected cell pellets (data not shown) and was similar to the levels illustrated in Fig. 4C.

**DISCUSSION**

Restriction of gp91phox promoter activity to mature myeloid cells is partially a consequence of a tightly controlled balance between a transcriptional repressor and transcriptional activators. The transcriptional repressor CDP binds to multiple sites within the proximal gp91phox promoter and excludes the binding of transcriptional activators (such as BID) to overlapping binding sites (7). CDP DNA binding activity is down-regulated during terminal myeloid cell differentiation, thus allowing activators to interact with the promoter. Previous studies demonstrated the importance of these regulated interactions. For example, constitutive overexpression of cloned CDP in a myeloid cell line prevents induction of gp91phox upon terminal differentiation (31). Furthermore, ablation of BID-binding sites within the −450 to −12 bp gp91phox promoter abrogates the ability of this promoter fragment to respond to INF-γ stimulation (9).

Binding of BID to the gp91phox promoter increases when nuclear extract derived from differentiated myeloid cells is analyzed by EMSA (9). However, because CDP also interacts with each of these promoter elements, increased BID binding could be due either to an increase in BID DNA binding activity, or to the decrease in CDP DNA binding activity that occurs during myeloid differentiation, or a combination of both. These studies were conducted to directly assess this question. In addition, the identification of BID permits an assessment of the
hypothesis that four distinct regions within the gp91phox promoter serve as binding sites for a common DNA-binding factor. Previously, this speculation was based on the similar mobility and binding site specificity exhibited in EMSA by each of the four identified BID complexes (8, 9).

Screening of an expression library with the −145 bp BID-binding site derived from the gp91phox promoter resulted in the recovery of a cDNA that encodes YY1. EMSA studies using YY1 antiserum demonstrates that each of the BID complexes contains YY1. Furthermore, an additional YY1-binding site was identified at −412 bp of the gp91phox promoter. Consistent with previous data demonstrating that ablation of BID-binding sites reduces gp91phox promoter activity (9), transient transfection studies demonstrate that YY1 trans-activates a minimal promoter containing the −145 bp YY1-binding site derived from the gp91phox promoter.

YY1 regulates multiple viral and cellular genes, although to our knowledge gp91phox represents the first lineage-restricted target of YY1 within the myeloid cell lineage. YY1 functions as a repressor, activator, or initiator depending on cell type, binding site, and interactions with other factors (see Ref. 32 for review). YY1 has also been reported to associate with the nuclear matrix (33). How YY1 activates transcription is not well understood. Interaction of YY1 with other proteins can affect the function of YY1 or its binding to DNA. YY1 has been shown to interact with several proteins including p300 (34), c-Myc (35), myeloid nuclear differentiation antigen (MNDa) (36), Sp1 (37), and E1A (38). The presence of E1A converts YY1 from a repressor to an activator of the AAV P5 promoter (30), and the interaction of YY1 and E1A is likely mediated by p300 (34). Although myeloid nuclear differentiation antigen has been reported to bind to YY1 and stimulate DNA binding activity (36), myeloid nuclear differentiation antigen antiserum failed to disrupt the BID complex that forms with the −145 Core-binding site probe (data not shown). Additionally, the BID complex exhibits a similar mobility and intensity in HeLa cells versus myeloid cells (Fig. 2A), thus myeloid nuclear differentiation antigen is not a component of the BID complex.

Direct examination of YY1 levels by Western blot analysis reveals that YY1 protein is not induced during phagocyte differentiation. However, this does not rule out the possibility of altered YY1 function during myeloid cell development, because YY1 activity may be post-translationally regulated by phosphorylation and/or ADP-ribosylation (39, 40). Importantly, formation of the BID complex with the −145 Core site probe, which lacks a CDP-binding site, is not increased in nuclear extracts derived from mature myeloid cells. In contrast, binding of YY1 to a promoter element containing a CDP-binding site increases in terminally differentiated myeloid cells that lack CDP DNA binding activity. This is consistent with previous data demonstrating latent BID activity in HeLa cells following the specific disruption of the CDP complex (7). This supports a model in which YY1 activity does not change during myeloid differentiation, but rather the ability of YY1 to interact with the gp91phox promoter is affected by the level of CDP DNA binding activity in the cell. The studies reported here thus reveal a novel regulatory mechanism for YY1, in which the access of the transcriptional activator YY1 to DNA-binding sites is controlled by modulation of a competing repressor DNA-binding protein (CDP). These results reinforce the conclusion that CDP is a critical regulator of the gp91phox promoter.

In addition to gp91phox, other genes that are specifically expressed in mature myeloid cells, such as the secondary granule genes lactoferrin and neutrophil collagenase, are also inhibited when CDP is overexpressed in differentiating myeloid cells (41, 42). This indicates that CDP regulates a panel of myeloid cell-restricted genes. Interestingly, inspection of the CDP-binding site within the lactoferrin promoter (42) reveals the presence of two putative YY1-binding sites (each exhibit an 8 of 9 bp match with the YY1 consensus binding sequence), which are separated by four nucleotides (data not shown). It will be of interest to determine if physical competition between CDP and YY1 is a recurring motif within myeloid cell-restricted gene promoters, and whether YY1 and CDP compete for binding to other promoter sites for which YY1 functions as an activator.

CDP competes with several transcriptional activators, such as BID/YY1, CP1, and IRF factors for binding to the gp91phox promoter (7). At other promoters, CDP interferes with the binding of additional transcriptional activators, including CP1, SATB1, C/EBP, Bright, hGCN5, ATF-1, TATA-binding factors, dbpA, and Phox2 (43–48). This report is the first demonstration of direct competition between CDP and YY1 for overlapping binding sites, although CDP and YY1 bind to distinct sites within the c-myc promoter (49, 50) and IgH Eμ intronic enhancer region (44, 51). Interestingly, the two YY1-binding sites surrounding the −225 and −90 bp sites of the gp91phox promoter contain IFN stimulated response element motifs that bind members of the IRF family (8, 9). IRF-1, IRF-2, and BID/YY1 each function as activators of the gp91phox promoter via the −90 bp element (8). IRF-1, IRF-2, and YY1 also bind to the IRG-47 promoter (52). In this case, however, YY1 is postulated to repress the IRG-47 promoter in uninduced cells and determine the magnitude of IRG-47 promoter expression in IFN-γ-induced cells (52).

CDP-binding sites have also been identified in many other promoters and enhancers for which competing transcriptional activators have not been identified (41, 42, 49, 53–57). In some cases CDP may not directly compete with activators for binding, and CDP-mediated transcriptional repression may be mediated via a carboxyl-terminal repressor domain (58). It was recently demonstrated that the CDP repression domain associates with histone deacetylase (43) thereby providing a possi-
YY1 as an Activator of the gp91phox Promoter

YY1 is an important activator of the gp91phox promoter. In the absence of CDP DNA binding activity, YY1 may contribute to the formation of an appropriate promoter or may associate with other DNA-binding factors. Members of the IRF family have been shown to interact or be functionally related to YY1. Physical interaction or functional cooperation between YY1 and CB6 promoter activity is down-regulated more generally upon terminal differentiation and exit from the cell cycle, and occurs in tissues in which it is distinct from physical displacement of activators.

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