Binding of Pleomorphic Adenoma Gene-like 2 to the Tumor Necrosis Factor (TNF)-α-responsive Region of the NCF2 Promoter Regulates p67phox Expression and NADPH Oxidase Activity*§

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NCF2, the gene encoding the NADPH oxidase cytosolic component p67phox, is up-regulated by TNF-α, and we recently mapped a region in the NCF2 promoter that was required for this TNF-α-dependent response. Because this TNF-α-responsive region (TRR) lacked recognizable transcription factor binding elements, we performed studies to identify factors involved in regulating NCF2 via the TRR. Using the TRR sequence as bait in a yeast one-hybrid screen, we identified the zinc finger transcription factor Pleomorphic Adenoma Gene-Like 2 (PLAGL2) as a candidate regulator of NCF2 expression. PLAGL2-specific antibodies were generated that detected the native and SUMO1-modified forms of endogenous PLAGL2. EMSA and DNA-binding protein affinity purification analyses demonstrated specific binding of in vitro-translated as well as endogenously expressed PLAGL2 to the TRR, and chromatin immunoprecipitation assays demonstrated enhanced binding of endogenous PLAGL2 to the TRR in vivo with TNF-α treatment. Knockdown of PLAGL2 protein inhibited up-regulation of NCF2 transcript, p67phox protein expression, and subsequent superoxide production in response to TNF-α. Furthermore, relative levels of native and SUMO1-modified endogenous PLAGL2 protein were modulated in a time-dependent manner in response to TNF-α treatment. These data clearly identify PLAGL2 as a novel regulator of NCF2 gene expression as well as NADPH oxidase activity and contribute to a greater understanding of the transcriptional regulation of NCF2.

The NADPH oxidase is a multicomponent, enzyme complex consisting of both membrane-bound and cytosolic components and is found in all phagocytic leukocytes, such as monocytes and neutrophils, where it plays an essential role in host defense against pathogens (reviewed in Ref. 1). Activation of the NADPH oxidase catalyzes electron transfer from NADPH to molecular oxygen, resulting in the production of superoxide anion (O2•−) (2). While O2•− is in itself not highly microbicidal, conversion of O2•− into more toxic reactive oxidants, such as hydrogen peroxide and hypochlorous acid, is required for pathogen killing (3).

The essential NADPH oxidase components include a membrane-bound flavocytochrome b558, which is a heterodimer of gp91phox and p22phox and the cytosolic proteins p47phox, p67phox, and p40phox (reviewed in Ref. 4). Additionally, activation of the NADPH oxidase requires the small GTPase Rac1/2 (5). Cell-free NADPH oxidase assays suggest flavocytochrome b558 and p67phox are sufficient for a functional oxidase; however, in vivo the system is clearly more complicated (reviewed in Ref. 6). Although the specific role of p67phox in enzyme function is still being determined, it has been proposed that p67phox is an NADPH-binding protein (7), and Dang et al. (8) subsequently showed that p67phox was able to directly bind NADPH. In addition, several groups have shown that p67phox binding to flavocytochrome b558 induces conformational changes in flavocytochrome b558 resulting in initiation of electron flow (9–11). The physiological importance of p67phox in NADPH oxidase function is demonstrated by certain forms of chronic granulomatous disease (CGD), which are caused by autosomal mutations in NCF2 (12). Patients with CGD have an inactive NADPH oxidase, making them susceptible to recurrent bacterial and fungal infections (reviewed in Ref. 13).

Regulation of p67phox expression appears distinct from other phox proteins, as p67phox is the last phox protein to be expressed during cellular differentiation, and its expression correlates the closest with the acquisition of oxidase activity (14). Thus, it has been proposed that p67phox may be the rate-limiting cofactor in NADPH oxidase activation (10, 15, 16). A number of studies indicate that cytokines and inflammatory mediators can modulate the level of expression of several oxidase proteins, including p67phox, in mature neutrophils and monocyte/macrophages via transcriptional regulation (17–19), and the identification of an increasing number of transcription factors involved in the regulation of NADPH oxidase gene expression demonstrates the complexity of this process (review in Ref. 20). To fully understand NADPH oxidase regulation, the identification of the complete repertoire of regulatory factors and characterization of the mechanisms whereby they regulate the NADPH oxidase is essential.
PLAGL2 Regulates the NCF2 TRR

We recently mapped a TNF$^2$-$\alpha$-responsive region (TRR) in the NCF2 promoter and showed that the TRR was essential for TNF-$\alpha$-induced up-regulation of p67$^\text{phox}$ (21). Although the TRR contained no apparent consensus binding sites for currently known transcription factors, DNase footprinting analysis demonstrated binding of nuclear factors to the NCF2 promoter in the region of the TRR (21). In the present study, we used the NCF2 TRR sequence in a yeast one-hybrid screen to identify transcription factors involved in NCF2 regulation. We identified a zinc finger transcription factor, Pleomorphic Adenoma Gene-Like 2 (PLAGL2), as an important regulatory factor that binds the NCF2 TRR and regulates NCF2 gene expression in response to TNF-$\alpha$.

EXPERIMENTAL PROCEDURES

Materials—Human TNF-$\alpha$ was from Research Diagnostics, Inc. (Flanders, NJ). Anti-TFIIB antibody was from Active Motif (Carlsbad, CA). 3-Amino-1,2,4-triazole (3-AT) was purchased from Sigma Co. Yeast S.D. minimal base media and amino acid supplements were from BD Biosciences (San Jose, CA). Unless otherwise noted, all oligomers and primers were from Integrated DNA Technologies (Coralville, IA). Anti-SUMO1 antibodies were from Abcam, Inc. (Cambridge, MA). TrueBlot secondary immunoblot antibodies (anti-rabbit or anti-mouse IgG) were from eBioscience (San Diego, CA). Alkaline phosphatase-conjugated and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were from Bio-Rad.

Cell Culture—MonoMac1 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate. HL60 cells were maintained in Dulbecco’s modified Eagles medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate.

Neutrophils and monocytes were purified from human blood collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils were purified using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as described previously (21). Monocytes were isolated by density gradient centrifugation, followed by Histopaque 1077 gradient separation and titrated with 0–50 mM 3-AT to determine stringency levels of the leaky HIS3 expression for the two clones Y187[pHisiLacZi(TRR)] and Y187[pHis-1pLacZii(TRR)]. 35 mM 3-AT was used to obtain the high-stringency clone Y187[pHis-1pLacZi(TRR)] and the low stringency clone Y187[pHisiLacZi(TRR)]. Additionally, transformed clones were assayed for auto-activity. The AH109 yeast strain was transformed with a human leukocyte cDNA library (Clontech) and selected on media lacking leucine. The bait strain (Y187) and the prey strain (AH109) were mated, and double-positive diploid clones were selected on media lacking histidine, uracil, and leucine, but supplemented with 35 mM 3-AT. Positive clones were restreaked on selective media and tested for $\beta$-galactosidase activity with an agar overlay assay. Clones positive for $\beta$-galactosidase activity for 24 h were then harvested. The prey plasmids from positive clones were extracted, amplified in E. coli, sequenced, and analyzed using the BLAST algorithm.

Cloning and Expression of PLAGL2—Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify PLAGL2 cDNA from human neutrophil RNA extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). Primer sequences of the PLAGL2 sense and antisense primers were 5'-GAATTCTAGGGCCCTGTGGACATGTT-3 and 5'-GAATTCTTGAACCCAGCTGTGAAAACG-3', respectively. Full-length PLAGL2 cDNA was cloned into the pcDNA3.1 eukaryotic expression vector, and the pcDNA3.1(PLAGL2) plasmid was used to express full-length PLAGL2 in the rabbit reticulocyte lysate TNT system (Promega, Madison, WI). The TNT product was immunoblotted using anti-PLAGL2 antibody to confirm the presence and correct size of in vitro transcribed and translated PLAGL2. Specificity of the band was established using peptide antigen as a blocking agent.

cDNA and RT-PCR Panel Screening—PLAGL2 gene expression was analyzed in adult human, fetal human, and human immune system cDNA panels (Clontech) using standard PCR conditions (30 cycles of 94 °C, 15 s, 55 °C, 30 s, 68 °C, 30 s). PLAGL2-specific primer sequences were: sense primer, 5’-CAGAGCCCGTGACATGTGTT-3’ and antisense primer, 5’-CATTACGTCGCCGAGAGGCC-3’. Expression was normalized to two housekeeping genes, $I\beta S$ and $GAPDH$.

Total RNA was isolated from cell lines and human neutrophils and monocytes, and cDNA was prepared, as previously described (21). PCR amplification was performed as above. PLAGL2 expression was normalized to previously amplified housekeeping genes $I\beta S$ and $GAPDH$ (21).

Anti-PLAGL2 Antibody Preparation and Characterization—A peptide corresponding to human PLAGL2 amino acid residues 50–68 (SNGEKLRPHSLPQPEQRPY) (Macromolecular Resources, Ft. Collins, CO) was conjugated to keyhole limpet hemocyanin (KLH, Sigma) using a 25% glutaraldehyde solution.

2 The abbreviations used are: TNF, tumor necrosis factor; TRR, TNF-$\alpha$-responsive region; EMSA, electrophoretic mobility shift assay; ds, double-stranded; ChIP, chromatin immunoprecipitation assay; PLAGL2, pleomorphic adenoma gene-like 2; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate.
(Sigma). KLH-peptide was used to immunize rabbits for polyclonal antibodies and mice for monoclonal antibodies. Monoclonal antibodies were obtained through fusion of the hyperimmune splenocytes with the myeloma line Sp2/0, as previously described (23). The fusion was screened against recombinant GST-PLAGL2 protein, and positive clones were subcloned by limiting dilution and rescreened by immunoblotting. Polyclonal anti-PLAGL2 IgG was purified from rabbit serum using Protein G-Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ). Monoclonal and polyclonal anti-PLAGL2 antibodies were characterized using recombinant PLAGL2-GST, rabbit reticulocyte in vitro translated PLAGL2, and human cell line extracts (HL60 and MonoMac1). Specificity of immunoblot analysis was confirmed using 5 μg/ml peptide antigen as a blocking agent. Unless otherwise indicated, rabbit polyclonal anti-PLAGL2 antibodies were used for immunoblot analysis and mouse monoclonal anti-PLAGL2 antibodies were used for immunoprecipitation.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitation was performed by preclearing 2 μg of MonoMac1 nuclear or cytoplasmic extract with TrueBlot anti-mouse IgG beads for 30 min at 4 °C and then incubating the samples with or without anti-PLAGL2 antibodies overnight at 4 °C. After addition of 50 μl of TrueBlot anti-mouse IgG beads, samples were incubated an additional 4 h at 25 °C, and the beads were washed three times with wash buffer (50 μM Tris-HCl, pH 8.0, 150 μM NaCl, 1% Nonidet P-40). Bound proteins were eluted by heating the beads for 5 min at 95 °C in SDS-PAGE sample buffer containing 50 μM dithiothreitol and analyzed by immunoblotting with polyclonal anti-SUMO1 and anti-PLAGL2 antibodies.

For p67phox immunoblot analysis, cells were seeded at 3 × 10⁵ cells/ml and treated with morpholine 36 h prior to harvest. At 12 h prior to harvest 20 ng/ml TNF-α was added. Protein was extracted using a CellLytic NuCLEAR extraction kit (Sigma), and cytosolic fractions were analyzed by immunoblotting with anti-p67phox polyclonal antibodies (24). Blots were quantified by densitometry.

Electrophoretic Mobility Shift Assay (EMSA)—Complementary oligonucleotides for the TRR and PLAG family consensus (PAGcon) sequences (see Fig. 4) were annealed by heating to 95 °C for 2 min and cooled to room temperature. Probes were end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase (Ambion, Austin, TX), purified using NucAway Spin columns (Ambion), and specific activity was determined.

Binding reactions were performed by preincubating 3 μl of rabbit reticulocyte-generated PLAGL2 protein for 30 min on ice in buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 μM ZnCl₂, 10% glycerol, 1 mM MgCl₂, 1 mM dithiothreitol, and 1 μg poly(dA-dT) or poly(dI-dC). 1 ng of end-labeled probe was added to the reaction mixture and incubated for an additional 30 min. For competition assays, unlabelled double-stranded (ds) DNA was incubated with the reaction mixture prior to addition of labeled probe. For supershift reactions, 10 μg of protein G purified, polyclonal anti-PLAGL2 antibody was added to the reaction mixture prior to addition of the labeled probe. Samples were run on NOVEX 6% DNA Retardation gels (Invitrogen) in TBE running buffer (89 mM Tris-Base, 89 mM boric acid, 2 mM EDTA, pH 8.3). Band shifts were visualized using autoradiography and quantified by densitometry.

Isolation of Nuclear Extracts—HL60 and MonoMac1 cells were seeded at 5 × 10⁶ cells/ml, allowed to recover for 9 h, and treated with 20 ng/ml TNF-α for the indicated times. Primary human monocytes were plated at 10⁵ cells/ml in RPMI on Primaria plates (BD Biosciences) precoated with fetal bovine serum, allowed to recover overnight, and treated with fresh RPMI containing 20 ng/ml TNF-α for the indicated times. Primary human neutrophils were plated at 5.5 × 10⁶ cells/ml RPMI on Primaria plates precoated for 1 h with 20% autologous serum and treated immediately with 20 ng/ml TNF-α.

For all cells, nuclear extracts were prepared using a CellLytic NuCLEAR extraction kit (Sigma), according to the manufacturer’s protocol. Lysis was performed in the presence of 20 mM N-ethylmaleimide (NEM) (Acros Organics, Geel, Belgium) for detection of PLAGL2. Nuclear extracts were snap-frozen in liquid nitrogen and stored at −80 °C.

DNA-binding Protein Affinity Purification—The bait sequence was prepared by ligation of the TRR sequence at 16 °C to obtain a TRR multimer of 11–23 repeats. 50 pmol of TRR multimer was ligated to the biotinylated oligonucleotide linker sequence bound to streptavidin-coated magnetic particles (Roche Applied Science) at 25 °C for 1 h. The TRR-coated particles were washed repeatedly and incubated for 2 h at 4 °C with 50 μg of HL60 cell nuclear extract in binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM ammonium sulfate, 1 mM dithiothreitol, 6.25 μg of poly(dI-dC), 0.63 μg of poly-L-lysine). The beads were washed, TRR-binding proteins were eluted with binding buffer containing 2 mM KCl for 30 min at 4 °C, and the eluates were analyzed by SDS-PAGE. TRR-binding proteins were detected via silver staining and immunoblot analysis using anti-PLAGL2 antibodies. Magnetic beads lacking the ligated TRR sequence were analyzed in parallel to determine background protein binding.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed using a ChIP-IT kit (Active Motif), according to the manufacturer’s protocol. Cells were seeded at 5 × 10⁵ cells/ml and treated with 20 ng/ml TNF-α for 12 h. The cells were harvested by centrifugation (4 × 10⁶ total cells), resuspended in RPMI containing 1% formaldehyde, and fixed for 10 min at room temperature. Nuclear extracts were prepared from the fixed cells, and enzymatic shearing of the chromatin was performed at 37 °C for 10 min, followed by addition of ice-cold EDTA to stop the reaction. Sheared chromatin was precleared with protein G beads prior to incubation overnight at 4 °C with 4 μg of anti-PLAGL2 antibody, negative IgG control antibody, or positive control antibody against TFIIB (Active Motif), which binds the GAPDH promoter. A sample of precleared chromatin was reserved for the input in PCR analysis. Antibody/DNA complexes were precipitated with protein G beads, washed, and eluted with a 1% SDS solution. RNA was digested with RNase A, and cross-links were reversed by overnight incubation at 65 °C. Protein was digested with Proteinase K for 2 h at 42 °C, and DNA was purified using the on-column purification method (Active Motif).

Purified, immunoprecipitated chromatin fragments were subjected to quantitative real-time PCR using SYBR Green dye
and an ABI 7500 Real Time PCR System, according to the manufacturer’s protocol for absolute quantification (ABI). 25 μl of immunoprecipitated DNA in a 50-μl reaction was subjected to 45 amplification cycles. The reserved input DNA was used to obtain the standard curve. PCR amplification of the NCF2 TRR was performed with primers flanking the TRR (sense primer, 5’-ATCTGGCCAGAAAGTGTA-3’, and antisense primer, 5’-CTTCATTCCAGAGGCTGATG-3’). Primers were optimized on ChIP input template DNA, and specificity was established with dissociation curves. All real-time amplifications were performed in triplicate, and antibody enrichment for the TRR sequence was calculated as % of DNA input. For comparison of transcription factor promoter binding efficiency between TNF-α-treated and untreated cells, anti-TFIIB immunoprecipitated DNA was amplified 40 cycles with primers to the GAPDH promoter (sense primer 5’-ATGTTATGGAAGGCCTCCTCAT-3’) and MO2 5’-CGCTGGTGAACATGTGTGCTAGG-3’). Anti-PLAGL2 immunoprecipitated DNA was amplified 40 cycles with primers to the TRR (see above). PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide.

**RESULTS**

**Identification of a TRR-binding Protein by Yeast One-hybrid Screening**—Previously, we found by DNase footprint analysis that nuclear proteins bind the NCF2 TRR; however, no identifiable binding elements for known DNA-binding proteins were apparent in the TRR sequence (21). To identify TRR-binding proteins, we utilized a yeast one-hybrid assay. The integrated bait sequence used for this assay was a trimer of the TRR bait sequence used for this assay was cloned into the yeast one-hybrid assay. The integrated bait sequence used for this assay was 3-AT with the two reporter strains, pHISi, pHis-1, and pLacZi (supplemental Fig. S1, panel A), and recombinant plasmids were integrated into the genome of the competent yeast strain Y187, combining pLacZi with either pHisi or pHis-1 in the same yeast clone creating double-reporter yeast strains. Additionally, the control integration and reporter vectors p53BLUE and p53HIS were linearized and independently integrated into the Y187 yeast genome.

The difference in the levels of leaky background of the pHISi and pHIS-1 integration and reporter vectors was used to design yeast one-hybrid assays of relative stringency. Using titrated levels of 3-AT with the two reporter strains, pHisiLacZi(TRR) and pHIS-1pLacZi(TRR), bait strains of two different stringencies were obtained. The Y187 [pHis-1pLacZi(TRR)] demonstrated high stringency with the leaky HIS3 expression sensitive to only 10 mM 3-AT, while the Y187 [pHisiLacZi(TRR)] strain was of lower stringency and required 45 mM 3-AT to completely inhibit the leaky background (supplemental Fig. S1, panel B). Additionally, all reporter strains were assayed for auto-activity (data not shown).

The competent reporter strain AH109 was transformed with a human leukocyte cDNA library fused to the GAL4 activation domain and subsequently mated with the bait strains at a density of 4.5 × 10^6 cells per cm². To confirm the mating strategy, the AH109 strain was transformed with the positive and negative control vectors pGAD53m and pGAD424, respectively, and diploids were screened for both nutritional and colorimetric gene activity (supplemental Fig. S1, panel C). The low stringency assay screened 1.7 × 10^7 library clones, while the high-stringency assay screened 4.6 × 10^7 library clones. Double-positive, diploid clones were selected on synthetic complete medium lacking histidine, uracil, and leucine and supplemented with 35 mM 3-AT. Of 64 diploid, double-positive (HIS/URA) clones recovered, 21 were from the high stringency
screen, and 43 were from the low-stringency screen. Twenty-seven of these clones tested positive for stable \(NCF2\)-galactosidase activity in an agar overlay assay (Fig. 1B). Of the 27 double-positives with \(NCF2\)-galactosidase activity, eleven diploids contained the sequence of a zinc finger transcription factor, Pleomorphic Adenoma Gene-like 2 (PLAGL2) (GenBank\textsuperscript{TM}/EBI accession number NM002657.2). These results implicated PLAGL2 as an \(NCF2\) TRR-binding protein.

Analysis of PLAGL2 Expression in Human Tissue, Leukocytes, and Cell Lines—The identification of PLAGL2 as a putative TRR-binding protein required that PLAGL2 be expressed in human phagocytes. We evaluated this issue here because initial studies suggested PLAGL2 was expressed in murine embryonic (25) and adult tissues (26), but only in human tissues during embryogenesis (25). In contrast, a subsequent study by Yang \textit{et al.} (27) using RT-PCR analysis indicated PLAGL2 was expressed in all human tissues, with higher expression observed in human lung tissues. Because of these inconsistencies, we used PCR to screen a series of human tissue cDNA panels for PLAGL2 expression and found PLAGL2 was ubiquitously expressed in all adult human tissues and almost all fetal human tissues, except for the relatively low level of expression observed in fetal brain (Fig. 2, A–C).

Although PLAGL2 expression has been reported in a mouse macrophage cell line (26), previous studies did not evaluate expression of PLAGL2 in the human immune system. Because the adult human cDNA panel indicated high levels of PLAGL2 expression in blood leukocytes (Fig. 2B), a human immune system cDNA panel was screened for PLAGL2 expression. Consistent with results observed in Fig. 2B, PLAGL2 was ubiquitously expressed, with relatively high expression in blood leukocytes (Fig. 2D). Further analysis of human primary immune cells and human immortalized cell lines for PLAGL2 expression showed that PLAGL2 message was present in primary phagocytes and a wide range of cell lines including myeloid and lymphoid lineages (Fig. 2E). Importantly, control samples containing no template demonstrated the absence of contamination (data not shown). Although the analysis was not performed quantitatively, there does appear to be a relatively higher level of...
PLAGL2 expression in human neutrophils versus the other cells tested.

Preparation and Characterization of Anti-human PLAGL2 Antibodies—A peptide corresponding to the published PLAGL2 amino acid sequence at residues 50–68 (CSNGEKLRPHSLPQPEQR) (supplemental Fig. S2) was synthesized and conjugated to KLH. This peptide antigen was chosen because of its minimal sequence similarity to other PLAG family members (28). Furthermore, this sequence is located between the first and second zinc finger regions of the N terminus, and these zinc fingers do not appear to be required for DNA binding or transcription (28, 29). Thus, antibody binding would likely not interfere with these events. KLH-conjugated peptide was used to immunize rabbits and mice to obtain polyclonal and monoclonal antibodies, respectively. Both monoclonal (data not shown) and polyclonal antibodies (Fig. 3A) stained endogenous PLAGL2 expressed in human MonoMac1 cells as well as in vitro-translated PLAGL2 produced in rabbit reticulocyte lysates. Furthermore, staining specificity was confirmed by addition of peptide antigen, which specifically blocked PLAGL2 staining (Fig. 3A).

Interestingly, immunoblots of MonoMac1 cell extract demonstrated the presence of two bands, and staining of both bands was blocked with peptide antigen (Fig. 3A). Because PLAGL2 has been shown to be modified by SUMO1 (30), we hypothesized that the two anti-PLAGL2 immunoreactive bands might correspond to the native and SUMO1-modified forms of PLAGL2, which have predicted mass of 55 and 68 kDa, respectively. To evaluate this issue, we immunoprecipitated PLAGL2 from MonoMac1 cell extracts with anti-PLAGL2 antibodies and blotted the immunoprecipitates with anti-PLAGL2 and anti-SUMO1 antibodies. Two immunoreactive bands, corresponding in size to the predicted native and SUMO1-modified PLAGL2, were observed in the anti-PLAGL2 immunoblot, and a band corresponding in size to a single SUMO1-modified PLAGL2 was observed in the anti-SUMO1 immunoblot (Fig. 3B). Thus, these data demonstrate our anti-PLAGL2 antibodies are specific for PLAGL2 and recognize both native and SUMO1-modified forms of the protein.

In Vitro Translated PLAGL2 Binds the NCF2 TRR—Electrophoretic mobility shift assay (EMSA) with in vitro-translated PLAGL2 was used to further investigate binding of PLAGL2 to the TRR. As seen in Fig. 4A (lane 1) PLAGL2 shifted the radio-labeled TRR probe, whereas preincubation with cold TRR double-stranded (ds) oligonucleotide completely eliminated this band shift (Fig. 4B, lane 2). Because the mapped TRR sequence does not share sequence similarity to the published PLAG family consensus binding sequence (PLAGcon) (25) (Fig. 4A), cold PLAGcon ds oligonucleotide was also used as a competitor for comparison. Preincubation of in vitro translated PLAGL2 with cold PLAGcon also completely eliminated the EMSA band shift, establishing that in vitro translated PLAGL2 binds both the TRR and the PLAGcon (Fig. 4B, lane 3). Furthermore, preincubation with an irrelevant, cold ds oligonucleotide did not compete away the EMSA band shift, indicating that in vitro translated PLAGL2 bound both the TRR and the PLAGcon in a sequence-specific manner (Fig. 4B, lane 4).

To further establish specificity of PLAGL2 binding to the TRR sequence, in vitro-translated PLAGL2 was preincubated...
with anti-PLAGL2 antibodies prior to addition of TRR probe. As shown in Fig. 4B the PLAGL2 band was supershifted by the anti-PLAGL2 antibody (lane 5) but not by a nonspecific antibody (lane 6).

The lack of similarity between the PLAGcon and TRR sequences prompted us to evaluate the relative affinity of PLAGL2 for these probes. Preincubation of PLAGL2 with increasing amounts of cold TRR prior to addition of labeled PLAGcon showed that PLAGL2 binding was reduced in a concentration-dependent manner and that 100-fold excess TRR completely blocked PLAGcon binding (Fig. 5A, lane 9). Likewise, addition of cold PLAGcon reduced binding of labeled TRR; however, PLAGL2 appears to bind with higher affinity, as only a 5-fold excess of cold PLAGcon was required to block binding of the labeled TRR probe (Fig. 5B, lane 5). Densitometric analysis of the autoradiographs confirmed that PLAGL2 has ~50-fold higher affinity for the PLAGcon sequence relative to the TRR sequence (Fig. 5C).

Native and SUMO1-modified Endogenous PLAGL2 Bind the TRR in Vitro—In conjunction with the yeast one-hybrid screen, we performed DNA-binding protein affinity purification to isolate TRR-binding proteins. Magnetic particles coated with TRR concatamers were incubated with HL60 cell nuclear extract, washed, and bound proteins eluted. Eluate was subjected to SDS-PAGE, followed by silver staining. The immunoblot analysis of proteins isolated by TRR DNA-binding protein affinity purification. Eluate from beads coated without (Control) or with (TRR) TRR multimers were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-PLAGL2 antibody. Two bands corresponding to the predicted M, of native and SUMO1-modified PLAGL2 are indicated with arrows.

In Vivo Binding of Endogenous PLAGL2 to the TRR Is Enhanced with TNF-α Treatment—Quantitative chromatin immunoprecipitation assay (ChIP) analysis with PLAGL2-specific antibody was used to determine if PLAGL2 binds the TRR in vivo. As shown in Fig. 7A, untreated MonoMac1 cells showed a significant enrichment of the TRR immunoprecipitated with PLAGL2 (anti-PLAGL2) as compared with mock immunoprecipitation (IgG). Furthermore, this enrichment was greatly enhanced by TNF-α treatment of the cells prior to cross-linking (Fig. 7A, right panel). To ensure that the enhanced enrichment with TNF-α was specific to the NCF2 TRR, PCR samples of the anti-PLAGL2 immunoprecipitated TRR and an anti-TFIIB immunoprecipitated GAPDH promoter sequence were run in parallel for both untreated and TNF-α-treated cells. Whereas a similar PCR amplification was observed for the GAPDH promoter sequence immunoprecipitated with anti-TFIIB antibody (Fig. 7B, lanes 1 and 2), the amplification of the TRR in TNF-α-treated cells was clearly enhanced when compared with that in untreated cells (Fig. 7B, lanes 3 and 4). Thus, these data demonstrate that endogenous PLAGL2 binds to the TRR in the NCF2 promoter in vivo and that this binding is enhanced by TNF-α treatment.

Knockdown of Endogenous PLAGL2 Inhibits the TNF-α Response in MonoMac1 Cells—Because of the toxicity of PLAGL2 overexpression in transfected MonoMac1 cells (data

FIGURE 5. Analysis of PLAGL2 affinity for PLAGcon and NCF2 TRR. A and B, in vitro translated PLAGL2 protein-DNA complexes (lane 2) were competed with cold PLAGL2con or cold TRR (lane 3). Competitors were preincubated with protein in increasing concentrations of 1-, 5-, 10-, 25-, 50-, and 100-fold excess of labeled probe (lanes 4–9 and lanes 13–18). The blot is representative of at least three experiments. C, PLAGL2 protein has enhanced binding affinity for the PLAGcon sequence. Densitometric analysis of a representative EMSA blot plotted as % binding relative to the uncompeted probe.

FIGURE 6. Native and SUMO1-modified PLAGL2 bind the TRR. A, analysis of proteins isolated by TRR DNA-binding protein affinity purification. Control magnetic particles and particles coated with TRR multimers (TRR) were incubated with HL60 cell nuclear extract, washed, and bound proteins eluted. Eluate was subjected to SDS-PAGE, followed by silver staining. The immunoblot analysis of proteins isolated by TRR DNA-binding protein affinity purification. Eluate from beads coated without (Control) or with (TRR) TRR multimers were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-PLAGL2 antibody. Two bands corresponding to the predicted M, of native and SUMO1-modified PLAGL2 are indicated with arrows.
PLAGL2 Regulates the NCF2 TRR

To confirm PLAGL2 protein expression was successfully knocked down, a knock-down strategy using morpholino oligonucleotides (31) was performed to determine if changes in PLAGL2 expression altered TNF-α-induced up-regulation of NCF2 in vivo. We designed one morpholino oligonucleotide to bind to a region of PLAGL2 mRNA just upstream of the start site (Fig. 8A, PL2 AS MO1) and one morpholino oligonucleotide to bind to a region of PLAGL2 mRNA flanking the ATG start site (Fig. 8A, PL2 AS MO2). A BLAST search with these morpholino sequences for nonspecific targets demonstrated there were no known targets in the human genome besides PLAGL2. Morpholinos encoding the inverse of these sequences were used as controls.

To establish effective delivery of morpholinos, FITC-labeled morpholinos were combined with EndoPorter and incubated with MonoMac1 cells. In general, diffuse fluorescence across the cell was observed, indicating successful permeabilization of the endosomal compartment and delivery to the cytosol (Fig. 8B, upper right panel). Transfection efficiency was determined to be >90%. Mock treatment with the delivery agent alone showed only background fluorescence with no cell staining (Fig. 8B, lower right panel).

To confirm PLAGL2 protein expression was successfully knocked down, immunoblot analysis was performed using MonoMac1 cell lysates after mock treatment or treatment with PLAGL2 antisense morpholinos. After 24 h of morpholino treatment, both the native and SUMO1-modified PLAGL2 proteins were knocked down to levels undetectable by immunoblot analysis (Fig. 8C). Using these experimental conditions, we analyzed the effect of PLAGL2 protein expression on TNF-α-induced up-regulation of NCF2 mRNA. Quantitative RT-PCR analysis in MonoMac1 cells demonstrated a ~7-fold increase in NCF2 mRNA levels in TNF-α-treated MonoMac1 cells compared with untreated cells (Fig. 8D). As shown in Fig. 8E, transfection with PLAGL2 antisense morpholino resulted in a statistically significant decrease in the TNF-α-induced fold change in NCF2 mRNA expression (Mock versus PL2 AS MO1 or PL2 AS MO2). In comparison, the TNF-α-dependent fold increase in NCF2 message in mock-treated cells was not significantly inhibited by transfection with inverted PLAGL2 morpholinos (Fig. 8E, Mock versus PL2 MO1in or PL2 MO2in). Increasing the morpholino treatment to 48 h did not significantly increase the knock-down effect on TNF-α-induced NCF2 message (data not shown). Thus, these results directly confirm PLAGL2 is required for the TNF-α-induced increase in NCF2 transcription.

To determine whether expression of p67phox protein correlated with the observed reduction in NCF2 message in PLAGL2 antisense morpholino-treated cells, we also analyzed p67phox expression by immunoblotting (Fig. 9A) and densitometric analysis (Fig. 9B) of the blots. As shown in Fig. 9, p67phox protein expression increased ~80% after TNF-α treatment, relative to the non-TNF-α control (NT+ versus NT−); whereas knockdown of PLAGL2 almost completely blocked the TNF-α-induced up-regulation of p67phox protein (MO1+/ versus MO1−). In contrast, treatment with the inverted PLAGL2 morpholino had no significant effect on p67phox expression (MO1in+/ versus MO1in−). Thus, these results confirm that PLAGL2 is required for TNF-α-induced up-regulation of p67phox protein.

Previous work in our laboratory showed a positive correlation between TNF-α-dependent up-regulation of NCF2 message and O2- production (21). Having shown that knockdown of endogenous PLAGL2 protein inhibits the TNF-α-induced up-regulation of NCF2 message and p67phox protein, we used the morpholino knock-down strategy to evaluate the effect of PLAGL2 on O2- production in response to TNF-α. In MonoMac1 cells, TNF-α treatment for 10 h increased PMA-induced O2- production ~3-fold (Fig. 10A); whereas morpholino antisense knockdown of PLAGL2 inhibited the TNF-α-dependent increase in O2- production, as compared with mock-treated cells (Fig. 10B). Importantly, the inverted morpholino control did not significantly affect O2- production, thus providing direct evidence that PLAGL2 is required for the TNF-α-dependent increase in O2- production observed in MonoMac1 cells.

TNF-α Modulates the Levels of Native and SUMO1-modified PLAGL2 Protein in MonoMac1 Cells and Human Primary Monocytes and Neutrophils—Based on the present data indicating PLAGL2 plays a role in regulating NCF2, we considered the possibility that this effect may be caused by the effects of TNF-α on PLAGL2 expression. Evaluation of PLAGL2 in TNF-α-treated MonoMac1 cells showed no significant change in PLAGL2 mRNA or protein levels relative to non-treated cells (supplemental Fig. S3). Recent studies suggested that SUMO1...
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PLAGL2 exhibited a biphasic expression pattern, decreasing for the first 3 h and then increasing in a time-dependent manner from 6–12 h after TNF-α treatment (Fig. 11A). Thus, these data indicate that TNF-α treatment affects the relative levels of the active (unmodified) and repressive (SUMO1-modified) forms of PLAGL2 protein in the nucleus and cytosol of MonoMac1 cells.

PLAGL2 was also analyzed in nuclear and cytosolic fractions isolated from TNF-α-treated human monocytes and neutrophils. In monocytes, TNF-α treatment induced a steady accumulation of native PLAGL2 in both the nuclear and cytosolic fractions, as well as a time-dependent accumulation of SUMO1-modified PLAGL2 in the cytosol (Fig. 11B). In contrast to MonoMac1 cells, there was no detectable SUMO1-modified PLAGL2 in monocyte nuclear fractions, even after 12 h of TNF-α treatment (Fig. 11B). It is possible, however, that SUMO1-modified PLAGL2 is present at levels below our detection limit. Neutrophils were also characterized by a unique pattern of PLAGL2 expression. As with MonoMac1 cells and monocytes, native PLAGL2 accumulated in a time-dependent manner in neutrophil nuclear fractions (Fig. 11C). SUMO1-modified PLAGL2 was also detected in nuclear fractions from untreated neutrophils and slowly decreased over time with TNF-α treatment (Fig. 11C).

**DISCUSSION**

Elevated TNF-α has been implicated in the pathology of numerous inflammatory syndromes, including septic shock, adult respiratory distress syndrome, rheumatoid arthritis, and Crohn’s disease, and anti-TNF-α treatment has proven effective in reducing the severity of these inflammatory diseases...
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FIGURE 9. Knockdown of PLAGL2 protein inhibits TNF-α-induced up-regulation of p67phox protein. A, untreated MonoMac1 cells (NT), cells treated with EndoPorter alone (Mock), and cells treated with EndoPorter and the indicated antisense morpholino (MO1in) or inverted morpholino (MO1in) for 24 h were treated with control buffer or TNF-α, as indicated. The cells were harvested, lysed, and cell extracts were immunoblotted with antibodies against p67phox. B, immunoblots were analyzed by densitometry, and data are presented as the relative % change in TNF-α-induced p67phox protein expression versus the corresponding non-TNF-α control sample.

FIGURE 10. Knockdown of PLAGL2 protein inhibits TNF-α-induced up-regulation of NADPH oxidase activity. A, TNF-α treatment of MonoMac1 cells enhances O2 production. Cells were treated with TNF-α for 10 h or prior to activation with 50 nM PMA, and the kinetics of O2 production were monitored by a chemiluminescence-based assay. Data are presented as relative levels of O2 (mean ± S.D.) generated in TNF-α-treated cells, with the untreated sample set to 1. B, knockdown of PLAGL2 protein inhibits TNF-α induction of O2 production. Cells were treated with EndoPorter alone (Mock) or EndoPorter and the indicated morpholinos for 24 h, followed by TNF-α treatment for 10 h. Cells were activated with 50 nM PMA, and the kinetics of O2 production was monitored, as described above. The data are presented as fold change in integrated chemiluminescence (mean ± S.D.) between TNF-α-treated samples and the untreated controls. Results are representative of three separate experiments, and statistically significant differences between TNF-α-treated samples and morpholino (PL2 AS MO1 and PL2 MO1in) + TNF-α-treated samples (*, p < 0.05) are indicated.

FIGURE 11. Analysis of PLAGL2 protein expression in MonoMac1 cells, primary monocytes, and primary neutrophils. Nuclear and cytosolic fractions were prepared in the presence of 20 mm NEM from MonoMac1 cells (A), monocytes (B), and neutrophils (C) after treatment with 20 ng/ml TNF-α for 12 h. Samples were analyzed by immunoblotting with anti-PLAGL2. The blots are representative of at least three experiments. Bands corresponding to native and SUMO1-modified PLAGL2 are indicated.

TNF-α signaling regulates the oxidative burst, the mechanisms behind TNF-α-induced NADPH oxidase activation remain elusive.

Previous work in our laboratory indicated that TNF-α may regulate the oxidative burst at the transcriptional level through regulation of NCF2 expression and, thus, expression of p67phox, a rate-limiting component of the NADPH oxidase (21). We mapped the sequence of the TRR in the NCF2 promoter (21) and, in the present study, identified the zinc finger transcription factor PLAGL2 as a TRR-binding factor. PLAGL2 is a recently discovered transcription factor that has been shown to play an important role in the pathogenesis of acute myeloid leukemia (37). However, little is known about this transcription factor and its endogenous role. To date, only a few potential PLAGL2 target genes have been identified (reviewed in Ref. 38); therefore, the demonstration of transcriptional regulation of NCF2 by PLAGL2 is a significant and novel finding. Binding of PLAGL2 to the TRR was implicated by the yeast one-hybrid screen, which indicated that PLAGL2 bound with high affinity to the TRR in yeast, as PLAGL2 was found in the majority of the double-positive interactions in the high stringency screen. Furthermore, the yeast one-hybrid results were supported in vitro using EMSA and DNA binding affinity purification assays and in vivo by ChIP analysis.

Binding of PLAGL2 to the TRR was surprising, as the PLAGcon sequence originally used to characterize the binding features of the PLAG family of transcription factors shares little sequence similarity to the TRR sequence (25). In examining the relative affinity of in vitro expressed PLAG2 for PLAGcon versus the TRR sequence, we found that PL2 had ~50-fold higher affinity for the PLAGcon sequence. Note, however, that

(continued)
the PLAGcon sequence was identified through random oligomer affinity purification and may not fully reflect the putative endogenous binding sequences for PLAG2. Indeed, PLAG2 was found to bind the hypoxia inducible factor-1 response element of the lactate dehydrogenase A promoter and, although the sequence of this element is also GC-rich, it does not contain the specific PLAGcon sequence (26). Interestingly, in the yeast one-hybrid assay, PLAG2 required the region containing the GC-rich sequence of the TRR (\(5\prime\)-GGCC-3\('\)) for binding (data not shown). Determining the specific PLAG2 binding element within the TRR will require further analysis.

Our PLAG2-specific antibodies recognized both native and SUMO1-modified forms of PLAG2, and both forms of PLAG2 bound specifically to the TRR sequence in vitro. SUMO modification has been reported to regulate the subcellular localization of substrates (39); however, the role of sumoylation in regulating PLAG2 localization remains controversial. Zheng and Yang (30) reported that SUMO1-modified PLAG2 localized to the nucleus in HEK293 cells. Although not directly demonstrated for PLAG2, Van Dyck et al. (40) reported that mutation of SUMO sites in PLAG1, a close family member, did not alter its nuclear distribution. Indeed, we detected SUMO1-modified PLAG2 in both cytosolic and nuclear fractions of MonoMac1 cells and neutrophils, but did not detect SUMO1-modified PLAG2 in nuclear extracts from monocytes. It is therefore unlikely that SUMO1 modification alone mediates nuclear-cytoplasmic localization of PLAG2.

SUMO1 modification has been shown to cause both activation and repression of transcription. For example, mutations at the site of sumoylation of the transcription factor Elk-1 or the coactivator p300 enhances transcription from responsive promoters, suggesting a repressive role for sumoylation (41, 42). Conversely, sumoylation of the heat shock factors HSF1 and HSF2 enhances transcriptional activity of these factors (43). GA-L4 DNA binding domain fusion protein assays were used to demonstrate that SUMO1 modification suppresses PLAG2 transactivation activity (30) and others demonstrated that sumoylation-deficient PLAG1 led to increased transactivation (40). Additional studies are required to determine if both forms of endogenous PLAG2 bind the TRR in vivo, and, if so, the effect they have on the regulation of NCF2 gene expression.

This is the first study to demonstrate the effects of TNF-\(\alpha\) on the relative levels of native and SUMO1-modified endogenous PLAG2 in the nucleus and cytoplasm. One possibility is that sumoylation may play a role in the regulation of TNF-\(\alpha\)-dependent \(p67^{phox}\) expression by modulating the activity of PLAG2 at the promoter. Modulation by sumoylation is involved in non-classical regulation of the TNF-\(\alpha\)-responsive transcription factor NF\(\kappa\)B, and with similar kinetics to those observed in our TNF-\(\alpha\)-treated lysates. In the classical pathway of TNF-\(\alpha\) activation of the NF\(\kappa\)B pathway, IkB\(\alpha\) is ubiquitin-ated and degraded, allowing NF\(\kappa\)B to move to the nucleus within minutes. However, sumoylation of IkB\(\alpha\) at the same lysine residue renders IkB\(\alpha\) resistant to TNF-\(\alpha\)-induced degradation and occurs slowly over several hours (44, 45). Additionally, direct modification of transcription factors, such as the glucocorticoid receptor, by sumoylation suggests a model in which SUMO modification results in modulation rather than a shut down of gene expression through synergy control motifs (46).

While our studies focused primarily on native and SUMO1-modified PLAG2, we cannot rule out a possible role for other post-translational modifications, such as acetylation (30) and phosphorylation (25). For example, Zheng and Yang (30) reported that sumoylation represses PLAG2 transactivation, whereas acetylation enhances PLAG2-mediated transactivation (30). Thus, multiple modifications may be utilized in PLAG2 regulation, and further studies are clearly needed to evaluate this issue.

In the present study, not only were both native and SUMO1-modified forms of PLAG2 shown to bind the TRR, but the binding of PLAG2 to the TRR was further enhanced with TNF-\(\alpha\) treatment. Significantly, knockdown of PLAG2 protein inhibited TNF-\(\alpha\)-induced up-regulation of NCF2 transcript, \(p67^{phox}\) protein, and \(O_{2}^{\cdot}\) production. Furthermore, TNF-\(\alpha\) treatment resulted in a time-dependent change in the relative levels of native versus SUMO1-modified PLAG2 in MonoMac1 cells, monocytes, and neutrophils. On the other hand, we observed no changes in PLAG2 message or protein levels in TNF-\(\alpha\)-treated MonoMac1 cells and no change in PLAG2 mRNA in TNF-\(\alpha\)-treated HL60 cells (data not shown). Thus, PLAG2 function appears to be determined by post-translational modification and the relative levels of active (unmodified) versus repressive (SUMO1-modified) PLAG2 in the nucleus. Accordingly, our data suggest that the modulation of PLAG2 localization in phagocytes plays an important role in regulating TNF-\(\alpha\)-dependent \(p67^{phox}\) expression and subsequent NADPH oxidase activity. Overall, a better understanding of the mechanisms involved in post-translational modification of PLAG2 by TNF-\(\alpha\) and subsequent effects on the NADPH oxidase activity may well prove to have significant therapeutic potential.

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