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Identification of a Minimal Cis-Element and Cognate Trans-factor (s) Required for Induction of *Rac2* Gene Expression During K562 Cell Differentiation

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Abstract

Rac2 is a Rho family GTPase that is widely expressed in hematopoietic cells and plays a critical role in host defense. This study investigates the mechanisms responsible for increased *Rac2* gene expression during myeloid cell differentiation. Treatment of K562 chronic myelogenous leukemia cells with phorbol-12-myristate-13-acetate (PMA) induces megakaryocytic differentiation and *Rac2* gene transcription following a lag of 6–12 hours. Promoter/luciferase reporter gene assays reveal that a 135 bp cis-element located between –4223 and –4008 bp upstream of the *Rac2* transcription start site is necessary and sufficient for PMA-induced gene expression. The AP1 transcription factor binds to three cis-elements within the 135 bp *Rac2* gene regulatory region both *in vitro* and *in vivo* following PMA treatment, and mutagenesis of the AP1 binding sites ablates the PMA responsiveness of the 135 bp *Rac2* gene regulatory region. Over-expression of AP1 is sufficient to induce expression of a transiently transfected *Rac2* promoter/luciferase plasmid, but not the endogenous *Rac2* gene. Induction of AP1 *in vitro* DNA-binding activity is apparent within 1 hour of PMA stimulation. However, AP1 binding to the endogenous *Rac2* promoter exhibits a lag of 5–9 hours, which correlates with reduced histone H3-Lys9 methylation, increased histone H3 acetylation, and increased nuclease accessibility within the 135 bp *Rac2* gene regulatory region. These results demonstrate that PMA induction of *Rac2* expression during terminal myeloid differentiation requires the coordinate induction of transcription factors and remodeling of *Rac2* gene chromatin structure.

Keywords

Transcription; Hematopoiesis; Epigenetics; Chromatin; Myeloid

1. Introduction

Rac proteins are members of the Rho GTPase family and function as molecular switches that cycle between a GTP-bound active state and a GDP-bound inactive state (Bourne et al., 1990). They are critical for several cellular processes such as cell proliferation, cytoskeletal organization, cell adhesion, cell polarity, chemotaxis, membrane trafficking, and

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transcriptional regulation (Huang et al., 1993; Laudanna et al., 1996; Erickson et al., 1997; Brown et al., 1998). Rac1, Rac2, and Rac3 proteins share greater than 90% amino acid identity but differ in their carboxyl-terminal sequences, which may confer distinct sub-cellular localizations and regulatory molecule interactions (Tao et al., 2002). Rac1 and Rac3 are ubiquitously expressed, while Rac2 expression is largely restricted to hematopoietic cells (Didsbury et al., 1989; Haataja et al., 1997)

The *Rac2* gene is located on chromosome 22q12 and consists of seven exons spanning 18 kb of DNA. Rac2 is abundantly expressed in myeloid cells and shows increased expression upon terminal myeloid cell differentiation (Didsbury et al., 1989). Rac2 accounts for greater than 90% of the Rac protein present in human neutrophils (Ambruso et al., 2000), and Rac2 plays a critical role in controlling cytoskeletal changes that are important for blood cell formation and function. Rac2 knock-out cells show defects in cortical F-actin assembly (Weston and Stankovic, 2004), defective integrin mediated cell adhesion, and enhanced mobilization (Yang et al., 2001). Rac2 is also important for assembly of the nicotinamide adenine dinucleotide phosphate oxidase complex that catalyzes the production of superoxide and plays a major role in host immune response (Knaus et al., 1991). A dominant-negative Rac2 mutation (D57N) in humans leads to impaired superoxide production by neutrophils (Gu et al., 2001) and recurrent infections (Williams et al., 2000). Lastly, *Rac2*^{-/-} mice show decreased chemotaxis and perturbed distribution of B and T lymphocytes (Yu et al., 2001; Croker et al., 2002). Thus, proper expression of Rac2 is critical for blood cell development and function. However, the molecular mechanisms regulating *Rac2* gene expression in blood cells have not been elucidated.

The proximal human *Rac2* gene promoter is GC-rich and lacks consensus TATA and CCAAT boxes but contains consensus binding sites for Sp1, Ets/PU.1, and MZF-1 (Ladd et al., 2004). The hematopoietic-specific expression of Rac2 has previously been investigated in cell lines and transgenic mice. The 4.5 kb proximal *Rac2* gene promoter region directs strong but promiscuous transcription in cell lines, suggesting that additional cis-elements are required to repress the *Rac2* gene in non-hematopoietic cells (Ladd et al., 2004). Consistent with this finding, the endogenous *Rac2* promoter is specifically de-methylated in hematopoietic cells, and pharmacologic de-methylation of the locus is sufficient to induce *Rac2* gene expression in non-hematopoietic cells (Ladd et al., 2004). A 30 kb bacterial artificial chromosome construct containing the human *Rac2* gene locus, including 1.6 kb of upstream sequence and 8 kb of downstream sequence, exhibits hematopoietic-specific expression in transgenic mice (Ladd et al., 2004).

Despite this previous work, regulatory elements involved in modulating *Rac2* gene expression during terminal hematopoietic cell differentiation remain to be identified. This study characterizes the molecular mechanisms that induce *Rac2* gene expression during phorbol-12-myristate-13-acetate (PMA)-stimulated megakaryocytic differentiation of human K562 chronic myelogenous leukemia cells. A 135 bp *Rac2* gene regulatory region is necessary and sufficient for PMA-induced transcription, and binds the PMA-responsive transcription factor AP1 following PMA-stimulated chromatin remodeling. These studies illustrate the complex interplay between regulation of chromatin structure and induced transcription factor expression that coordinately modulate Rac2 expression during myeloid cell differentiation.

2. Materials and Methods

2.1. Cell culture and transfections

K562 human chronic myelogenous leukemia cells were grown in suspension in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 5% fetal clone III serum (Hyclone, Logan, UT), 0.2 mM glutamate, and 50 units/ml penicillin/streptomycin at 37°C and

5% CO₂. Megakaryoblastic differentiation of K562 cells was induced with 100 nM PMA (Sigma Chemical Co., St Louis, MO) dissolved in dimethyl sulfoxide (DMSO), or alternatively K562 cells were treated with 100 nM of the inactive analog phorbol-12,-13-didecanoate (PDD) (Sigma Chemical Co., St Louis, MO) as a negative control.

Plasmid constructs were transiently transfected into 1×10^7 K562 cells in 300 μ l of culture medium by electroporation with a voltage of 220 V and capacitance of 950 μ F. Transfected cells were divided into two samples. One sample was treated with 100 nM PMA and the other with DMSO. Cells were harvested at 24 hrs post-transfection and lysed. Luciferase assays were performed using the luciferase reporter assay system (Promega, Madison, WI, USA) as per the manufacturer's protocol and were read using a Lumat LB 9501 chemiluminometer (EG & G Berthold, Wildbad, Germany). The assays were performed in triplicate at least three times.

For over-expression studies, 1×10^7 K562 cells were mixed with 0.5 μ g of cytomegalovirus (CMV)- β -galactosidase plasmid, 2.5 μ g of *Rac2* promoter construct, and 8 nanomoles of pcDNA3.1 (Invitrogen, Carlsbad, CA), pcDNA3.1/c-Jun, pCMV6-XL5 (Origene Technologies, Rockville, MD), pCMV6-XL5/c-FOS, or the c-Jun and c-Fos expression vectors plus empty pBluescriptSK+ vector for a total of 40 μ g of DNA. Cells were harvested 24 hrs post transfection, lysed, and analyzed using luciferase and β -galactosidase assays. Beta-galactosidase assays were performed as described by Sambrook et al. (1989).

2.2. Plasmid construction

The EF1 α promoter from plasmid pEF1 α /PAC (kindly provided by Dr. Mary Dinanuer, Indiana University) was subcloned into the pGL3-Basic luciferase reporter gene vector (Promega, Madison, WI). 5'-deletion constructs of the 4.5 kb human *Rac2* gene promoter were subcloned into pGL3-Basic. Truncations were created using restriction enzymes *Stu*I at -4223 bp, *Afl*III at -4088 bp, *Hind*III at -3824 bp, and *Kpn*I at -1100 bp. Two additional constructs were made by subcloning PCR fragments spanning -3500 bp to +134 bp and -30 to +134 bp (core *Rac2* promoter) into pGL3-Basic. Additionally, the 135 bp *Rac2* gene regulatory region between -4223 to -4088 bp was PCR amplified and subcloned upstream of the core *Rac2* promoter region in the pGL3-Basic vector. The full-length human c-Jun cDNA (kindly provided by Dr. Alexander Dent, Indiana University) was subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The full-length human c-Fos cDNA in the pCMV6-XL5 expression vector was purchased from Origene Technologies (Rockville, MD).

2.3. Quantitative real-time PCR

Total RNA was extracted from K562 cells using Tri-reagent (Molecular Research Center, Cincinnati, OH). Two hundred nanograms of RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers (Roche, Mannheim, Germany) as per the manufacturer's instructions. All quantitative real-time PCR reactions were performed in a 25 μ l mixture containing 20 ng of cDNA preparation (2 μ l), SYBR-Green I core reagent, including AmpliTaq-GOLD polymerase (Applied Biosystems, Foster City, CA), and 0.4 μ M primers. Real-time PCR analysis was performed for human *Rac2* mRNA and human Actin mRNA, and relative quantification was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was carried out using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). *Rac2* transcript levels were normalized to Actin transcript levels from the same preparations of cDNA.

2.4. Flow cytometric analysis

K562 cells treated with DMSO, PMA, or PDD were collected and washed twice with 1X PBS. Viable cells were counted by trypan blue exclusion, and 1×10^5 cells per tube were resuspended in 1X PBS and labeled with PE-conjugated CD41 antibody (kindly provided by Dr. Mary

Dinauer, Indiana University). Mouse IgG2A (kindly provided by Dr. Edward F. Srouf, Indiana University) was used as an isotype control. Flow cytometry was performed on a FACScan Analyzer (Becton Dickinson, San Jose, CA). Histograms are based on the analysis of 10,000 events.

2.5. Site-directed mutagenesis

Mutation of AP1 binding sites within the -4223 to -4088 bp (135 bp) regulatory region of the *Rac2* gene was performed with the Quick change II site-directed mutagenesis kit (Stratagene, La Jolla, California) using the following oligonucleotide primers (sense strand shown) - Oligo 1: 5'- ccctgtctgtgtgtgttcgagaccccgagcgcgcag -3', Oligo 4: 5'- tgaataaccaaatgcaagctctccctgcggtgggac -3', Oligo 7: 5'- ggcgagcaccacgaagcctcgaaccactcaacgtttcac -3'. The mutated nucleotides are underlined. Oligonucleotides were synthesized by Operon Biotechnologies Inc (Huntsville, AL). The amplified products were digested with *DpnI* followed by transformation into XL1-Blue supercompetent cells. The transformants were then analyzed for the insertion of each mutation by sequencing (DNA sequencing core facility, Indiana University School of Medicine). Mutations incorporated into the 135 bp *Rac2* gene regulatory region were subcloned upstream of the -30 to +134 bp *Rac2* core promoter in the pGL3-Basic reporter gene vector.

2.6. Electrophoretic mobility shift assay

Nuclear extracts were isolated from unstimulated and PMA-stimulated K562 cells using a modification of the Dignam protocol (Dignam et al., 1983). Protein concentrations were determined by Bradford assay (BioRad, Richmond, California). Double strand oligonucleotide probes were end-labeled with γ -³²P-ATP using T4 polynucleotide kinase followed by purification with Sephadex G-50 spin columns (Roche, Mannheim, Germany). For electrophoretic mobility shift assays (EMSAs), 5 μ g of nuclear extract was incubated on ice with 1×10^4 CPM radiolabeled oligonucleotide, 20 μ l binding buffer (10 mM Tris pH 7.5, 50 mM K-glutamate, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 5% glycerol) and 0.5 mM poly dI-dC. In reactions using competitor DNA, a 70-fold molar excess of non-radiolabeled double-strand competitor oligonucleotide was added and incubated on ice for 30 min. For supershift analysis, 2-3 μ g of antiserum raised against unmodified c-Jun (Active Motif, Carlsbad, CA) or c-Fos (Santa Cruz Biotechnologies, Santa Cruz, California) was added, followed by incubation on ice for 20 min and room temperature for 20 min.

Oligonucleotides used in EMSA analysis include (only one strand shown): AP1 control oligo, 5'- gcttgatgatgactcagccggaa -3' (Sawai et al., 1995); AP1 mutant oligo, 5'- cgcttgatgacttgccggaa -3' (Sawai et al., 1995); Oligo 1, 5'- gttgttacatgtgtcatgtgtgtctgtcttt -3'; Oligo 2, 5'- gtgtctgtccctttattagtgagtagtgttt -3'; Oligo 4, 5'- ggatgtgtcacatttggtattcatttagttt -3'; Oligo 5, 5'- ttcatttagtcccatctcttttgactacattt -3'; Oligo 6, 5'- ttgactacatttgcaaacactgaatcttt -3'; and Oligo 7, 5'- tcaactgaatcacagaaccacgagcctctgttt -3'. The underlined nucleotides denote consensus AP1 binding sites in the AP1 control oligo, Oligo 1, Oligo 4, and Oligo 7, and the mutated AP1 site in the AP1 mutant oligo. Samples were subjected to electrophoresis on 5% polyacrylamide gels in 0.5X Tris-borate EDTA at 4°C. Gels were dried and exposed to Kodak (Rochester, NY) Hyperfilm at -80°C with an intensifying screen.

2.7. Chromatin immunoprecipitation assay

Chromatin preparation and immunoprecipitations were performed using a ChIP kit (Millipore, Bedford, MA) with minor modifications of the manufacturer's protocol. Protein-DNA crosslinking was achieved by incubating K562 cells (2×10^6 cells/reaction) with 1% formaldehyde for 10 min at 37°C. Crosslinking was blocked by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 5 min. Cells were washed and lysed, and DNA was sonicated to an average length of 0.2-1 kb. The sonicated lysates were

then cleared by centrifugation and diluted 10-fold with ChIP dilution buffer provided in the kit. One percent of the diluted lysate was reserved as an input sample. Chromatin was immunoprecipitated with antibodies directed against RNA polymerase II (Santa Cruz), c-Jun (Santa Cruz), c-Fos (Santa Cruz), acetylated histone H3 (Millipore, Bedford, MA), dimethylated histone H3 K9 (Millipore), or normal rabbit serum (Santa Cruz). Immune complexes were collected using Protein A agarose (Millipore), and washed and eluted with freshly prepared elution buffer (1% SDS and 0.1 M NaHCO₃). The DNA was reverse crosslinked using 5 M NaCl and column purified using a high pure PCR product purification kit (Roche, Mannheim, Germany). The 135 bp human *Rac2* gene regulatory region between -4223 and -4088 bp was detected by standard PCR using the following primers: 5'-actttgcgttttctaggatttcac-3' and 5'-caagcattgtctatcaatggcac-3'. A 129 bp intragenic region (+15 kb) of the human *Rac2* gene locus was detected with the following primers: 5'-ttcacttcagggaactgtgc-3' and 5'-gcacagatgaggagaaaaggc-3'. A 210 bp intragenic region (+3.5 kb) of the human *Actin* gene locus was detected with the following primers: 5'-ggtgatagcattgcttctgtg-3' and 5'-gtctcaagtcagtgtacaggt-3'.

2.8. Nuclease accessibility assay

Nuclei were isolated from K562 cells treated with DMSO or 100 nM PMA using nuclei preparation buffer (15 mM Tris-HCl pH 7.5, 0.3 M sucrose, 60 mM KCl, 2 mM ethylene glycol tetraacetic acid [EGTA], 15 mM NaCl, and 5 mM MgCl₂) containing 5% NP-40. Further washes were performed in nuclei digestion buffer without NP-40 and samples were stored in nuclei preparation buffer at -80°C until used. Aliquots of nuclei (1×10⁶) were incubated with 100 U of Micrococcal nuclease S7 (MNase) (Roche) in nuclei digestion buffer (60 mM CaCl₂ and 750 mM NaCl) for 20 min at 37°C, and the reactions were terminated by the addition of stop buffer (10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.5% SDS, and 100 µg/ml proteinase K). Genomic DNA was purified using the high pure PCR product purification kit (Roche). The 135 bp *Rac2* gene regulatory region was PCR amplified and PCR product intensities were determined using ImageJ software (NIH). The amount of PCR product amplified from samples digested with MNase was normalized to the amount of PCR product amplified from undigested DNA.

3. Results

3.1. *Rac2* gene expression increases upon PMA stimulation and megakaryocytic differentiation of K562 cells

Treatment of K562 cells with PMA results in changes of cell morphology and acquisition of cell surface markers unique to megakaryocytes (Burger et al., 1992; Racke et al., 1997). K562 cells became adherent following 3 days of PMA treatment (Fig. 1A), indicating increased cell-surface adhesion (Whalen et al., 1997). These effects were not seen either following treatment with PDD, an inactive analog of PMA, nor with DMSO vehicle (Fig. 1A). Further studies were performed to examine expression of platelet surface glycoprotein IIb/IIIa (CD41 antigen), an early marker of megakaryocyte differentiation (Burger et al., 1992). Flow cytometric analysis showed increased CD41 expression in K562 cells stimulated with PMA compared to cells treated with DMSO or PDD (Fig. 1B). *Rac2* expression was previously reported to be up-regulated upon terminal myeloid cell differentiation (Didsbury et al., 1989). The level of *Rac2* mRNA during K562 cell differentiation was examined by quantitative real-time PCR analysis. PMA stimulation led to a 5 to 6-fold increase in *Rac2* mRNA expression after 24 hrs of treatment compared to that of cells treated with DMSO. Cells treated with PDD did not show a significant change in *Rac2* gene expression (Fig. 1C). Induction of *Rac2* gene expression was further confirmed by RNase protection assay, which similarly demonstrated a 6-fold increase in *Rac2* mRNA levels upon PMA stimulation of K562 cells for 24 - 48 hrs (data not shown).

Quantitative real-time PCR analysis revealed that *Rac2* mRNA levels remain unchanged for at least 6 hrs following PMA stimulation, followed by a time-dependent increase at 12 and 24 hrs of treatment (Fig. 2A). Chromatin immunoprecipitation analysis was used to determine if increased *Rac2* mRNA levels is a consequence of increased transcription. Consistent with the kinetics of *Rac2* mRNA induction, treatment of K562 cells with PMA resulted in a progressive enrichment of RNA polymerase within the coding region of the *Rac2* gene following an initial lag period of at least 6 hrs, indicating an increase in the rate of transcription at the 12 and 24 hr time points (Fig. 2B). No enrichment of RNA polymerase II within the *Actin* gene was observed in K562 cells treated with PMA.

3.2. PMA-responsive regulatory cis-elements reside within the 4.5 kb proximal *Rac2* gene promoter

A luciferase reporter plasmid carrying 4.5 kb of the proximal *Rac2* promoter (-4358 to +134 bp) was transiently transfected into K562 cells. Following transfection the cells were split into two populations. One sample was treated with PMA and other with DMSO. PMA stimulation resulted in a 5-fold increase in reporter gene expression compared to that of cells treated with DMSO (Fig. 3A). This is similar to the induction of endogenous *Rac2* mRNA levels observed upon treatment of K562 cells with PMA (Fig. 1C). An SV40 promoter/luciferase vector was used as a positive control and an EF1 α promoter/luciferase vector was used as a negative control. These data demonstrate that cis-elements sufficient to direct PMA-responsive *Rac2* gene expression reside within the proximal 4.5 kb *Rac2* promoter region.

3.3. A 135 bp region within the 4.5 kb proximal *Rac2* gene promoter is necessary and sufficient for the induction of transcription upon PMA stimulation

A series of 5' deletions of the 4.5 kb *Rac2* promoter were generated to identify the cis-elements necessary for PMA induction of *Rac2* gene expression in K562 cells following transient transfection (Fig. 3B). Deletion of the promoter to -4223 bp did not alter the level of reporter gene induction compared to that of the 4.5 kb construct. However, deletion of a 135 bp region between -4223 and -4088 bp led to the complete abolishment of PMA-induced promoter activity (Fig. 3B). The basal unstimulated transcriptional activity of these constructs did not show any significant variation (data not shown).

The 135 bp *Rac2* gene regulatory region (-4223 to -4088 bp) was subcloned upstream of the -30 to +134 bp *Rac2* core promoter in the pGL3-luciferase reporter gene vector (referred to as 135 bp + core *Rac2*/luciferase) to test if this regulatory region is sufficient to serve as a PMA-responsive cis-element. Transient transfection of this construct showed a significant increase in the induction of reporter gene expression compared to that of the core *Rac2* promoter construct (Fig. 3C). Taken together, these results demonstrate that the 135 bp *Rac2* regulatory region is necessary and sufficient to direct increased transcriptional activity of the *Rac2* gene promoter in response to PMA treatment.

3.4. Identification of PMA-responsive DNA-binding proteins that interact with the 135 bp *Rac2* gene regulatory region

EMSA was performed with seven overlapping oligo probes that span the 135 bp *Rac2* regulatory region to detect PMA-induced DNA-binding proteins (Fig. 4A). The Oligo 1, 4, and 7 probes, each of which contains an AP1 consensus binding site as revealed by the TRANSFAC transcription factor search analysis program (Wingender et al., 1996; Wingender et al., 1997), produced a DNA-protein complex that becomes more intense following stimulation with PMA. Although nuclear proteins bind to all of the probes, no difference in protein binding was observed upon PMA stimulation for Oligo 2, 3, 5, or 6 probes (Fig. 4B). In addition, the PMA-responsive protein binding observed with probes 1, 4, and 7 were of similar mobility when run

on the same gel (data not shown), suggesting that a common transcription factor may occupy three distinct sites within the 135 bp *Rac2* gene regulatory region.

Competition experiments were performed to further characterize the transcription factor(s) that binds to Oligo 1, 4, and 7. Each of the PMA-responsive mobility shifts observed with Oligo 1, 4 and 7 probes are effectively disrupted by unlabeled Oligo 1, 4, and 7 competitors (Fig. 4C). This result demonstrates that transcription factors with similar binding sequence specificity interact with all three sites within the 135 bp *Rac2* gene regulatory region.

AP1 consists of homo- or heterodimers of Jun, Fos, or ATF proteins (Bohmann et al., 1987; Hess et al., 2004). In addition, Jun and Fos are early response genes that play important roles in myeloid cell differentiation (Lord et al., 1993; Shafarenko et al., 2004). To further assess whether AP1 binds to all three sites within the 135 bp *Rac2* regulatory region, an unlabeled AP1 control oligo known to bind AP1 (Ignatov and Keath, 2002) was added to the EMSA reaction as a competitor. The non-radiolabeled AP1 oligo competitor effectively disrupts the PMA-responsive protein binding seen with all three probes (Fig. 4C). Addition of non-radiolabeled mutant AP1 oligo, which fails to bind AP1 (Zutter et al., 1999; Lai and Cheng, 2002), did not disrupt the PMA responsive protein binding (Fig. 4C). Furthermore, when used as a probe, the AP1 control oligo generates a retarded band of similar mobility as that observed with the Oligo 1, 4, and 7 probes (Fig. 4C).

Addition of anti-c-Jun and anti-c-Fos antibody resulted in supershift of the complexes generated with Oligo 1, 4 and 7 probes (Fig. 4D). No alteration in complex formation was observed following addition of IgG (data not shown). In aggregate, these results demonstrate that AP1 binds to three distinct sites within the 135 bp *Rac2* gene regulatory region.

3.5. AP1 binds to the 135 bp *Rac2* regulatory region in vivo

Chromatin immunoprecipitation assays were conducted to assess AP1 binding to the *Rac2* gene promoter *in vivo*. Crosslinked and sheared chromatin prepared from unstimulated K562 cells and K562 cells treated for 24 hrs with PMA was immunoprecipitated with c-Jun or c-Fos antibodies. Figure 5 demonstrates that both c-Jun and c-Fos bind to the 135 bp *Rac2* regulatory region *in vivo* upon PMA treatment of K562 cells, but not in K562 cells treated with DMSO. The interaction of AP1 with the *Rac2* regulatory region promoter is specific, as no enrichment of AP1 is observed within the body of the *Rac2* gene (+15 kb) that lacks consensus AP1 binding sites (Fig. 5).

3.6. All three AP1 sites within the 135 bp *Rac2* regulatory region regulate promoter activity upon PMA stimulation

Each of the three AP1 sites within the 135 bp + core *Rac2*/luciferase construct was individually ablated by site-directed mutagenesis. Ablation of any of the AP1 sites led to a significant decrease in reporter gene activity, compared to that of the wild type construct, following transient transfection and PMA stimulation (Fig. 6C). Triple mutation of all three AP1 binding sites completely abolishes PMA-induction of reporter gene activity (Fig. 6A). These results demonstrate that all three AP1 sites within the 135 bp *Rac2* regulatory region contribute to the transcriptional activity of the *Rac2* promoter following PMA stimulation.

3.7. Trans-activation of *Rac2* promoter activity by AP1

To determine if AP1 is sufficient to trans-activate the *Rac2* promoter, K562 cells were co-transfected with the 135 bp + core *Rac2*/luciferase construct and AP1 expression vectors. c-Jun proteins can form stable homo-dimers but bind DNA less effectively than c-Jun /c-Fos heterodimers (Bakiri et al., 2002). In addition, c-Jun homodimers can serve as co-activators of transcription (Grondin et al., 2007). However, over-expression of c-Jun failed to trans-activate

Rac2 promoter activity (Fig. 6B). On the other hand, c-Fos only forms heterodimers with c-Jun and cannot form homodimers to activate transcription (Milde-Langosch, 2005). As expected, co-transfection of a c-Fos expression construct also fails to trans-activate the *Rac2* promoter. However, co-transfection of both c-Jun and c-Fos expression vectors led to a 6.5-fold increase in reporter gene expression compared to cells co-transfected with empty vector (Fig. 6B). Over-expression of c-Jun and c-Fos proteins in transfected K562 cells was confirmed by western blot analysis (data not shown). These results demonstrate that c-Jun/c-Fos AP1 heterodimers are sufficient to activate transiently-transfected *Rac2* promoter constructs in the absence of PMA stimulation.

Additional studies were performed to determine if expression of exogenous AP1 similarly induces expression of the endogenous *Rac2* gene in the absence of PMA stimulation of K562 cells. Twenty four hours after co-transfection with c-Jun and/or c-Fos expression vectors, quantitative real-time PCR analysis was performed to examine endogenous *Rac2* gene expression. In contrast to the behavior of transiently transfected *Rac2* promoter constructs, over-expression of AP1 in K562 cells failed to induce expression of the endogenous *Rac2* gene in the absence of PMA stimulation (Fig. 6C).

Because exogenous AP1 trans-activates transiently transfected *Rac2* promoter constructs but not the endogenous *Rac2* gene, additional studies were performed to compare the kinetics of *in vitro* AP1 DNA-binding activity to the Oligo 1, Oligo 4, and Oligo7 probes derived from the 135 bp *Rac2* regulatory region following PMA stimulation. Consistent with a previous report (Eriksson et al., 2005), EMSA analysis reveals that AP1 DNA-binding activity is induced within 1 hr of PMA stimulation of K562 cells (Fig. 7). Given that there is a 6-12 hr lag following PMA stimulation before *Rac2* gene induction (Fig. 2), these data reveal that induction of AP1 DNA-binding activity is not sufficient to induce expression of the endogenous *Rac2* gene.

3.8. PMA stimulation induces chromatin remodeling at the 135 bp *Rac2* gene regulatory region

Further studies were conducted to examine whether regulated chromatin structure correlates with the ability of AP1 to bind to the 135 bp *Rac2* regulatory region *in vivo*. The functional organization of chromosomes into euchromatin and heterochromatin domains is associated with distinct post-translational modifications including acetylation and methylation of histone tails (Cheung et al., 2000; Jenuwein and Allis, 2001). To determine if histone modifications change within the 135 bp *Rac2* regulatory region following a 24 hr PMA stimulation of K562 cells, chromatin immunoprecipitation assays were performed using antibodies specific for histone H3K9 dimethylation, a marker of inactive chromatin (Nakayama et al., 2001; Peters et al., 2002; Tamaru et al., 2003), and histone H3 K9/14 acetylation, a marker of active chromatin (Jenuwein and Allis, 2001; Roh et al., 2005). Immunoprecipitated genomic DNA enriched for these modifications was then analyzed by PCR for the presence of the 135 bp *Rac2* regulatory region. PMA-stimulated K562 cells showed a marked decrease of histone H3K9 dimethylation within the 135 bp *Rac2* regulatory region compared to unstimulated cells. In contrast, upon PMA stimulation of K562 cells there was a striking increase of histone acetylation within the 135 bp *Rac2* gene regulatory region (Fig. 8A).

Chromatin modifications associated with transcriptionally active DNA, such as histone acetylation, recruit chromatin remodeling complexes, thereby changing the physical structure of the chromatin to a more open form (Struhl, 1998; Sterner and Berger, 2000; Gorisch et al., 2005). Nuclease sensitivity assays were performed to assess chromatin accessibility within the 135 bp *Rac2* regulatory region. Intact nuclei isolated from K562 cells stimulated with PMA or treated with DMSO were treated with MNase, followed by PCR amplification of the 135 bp *Rac2* regulatory region. These experiments reveal that the 135 bp *Rac2* gene regulatory region isolated from PMA-stimulated K562 cells is more accessible to MNase compared to cells

treated with DMSO (Fig. 8B). This indicates that PMA stimulation of K562 cells leads to a more relaxed chromatin structure within the 135 bp *Rac2* regulatory region.

To determine the kinetics of histone H3 acetylation and AP1 binding within the 135 bp *Rac2* regulatory region following PMA stimulation of K562 cells, ChIP assays were performed using antibodies directed against c-Jun and histone H3 acetylation. As shown in figure 8C, both histone acetylation and binding of c-Jun to the 135 bp *Rac2* regulatory locus starts to increase following 5 hrs of PMA treatment and are markedly increased by 9 hrs of PMA treatment (Fig. 8C). The kinetics of histone acetylation and c-Jun binding within the 135 bp *Rac2* regulatory region fits well with the kinetics of *Rac2* mRNA induction (Fig 3A). In addition, the kinetics of chromatin remodeling of the 135 bp *Rac2* regulatory locus was similarly analyzed by MNase sensitivity assay. Intact nuclei isolated from K562 cells treated with PMA for various time periods were digested with MNase. PCR amplification of the 135 bp *Rac2* regulatory region reveals a dramatic increase in MNase sensitivity following 9 and 24 hrs of PMA treatment (Fig. 8D). These results are consistent with the model that changes in the chromatin structure of the 135 bp *Rac2* gene regulatory region are required to permit binding of AP1 to induce *Rac2* gene expression.

4. Discussion

The objective of this study was to investigate the genetic and epigenetic regulation of the *Rac2* gene during myeloid cell differentiation. PMA-stimulated megakaryocytic differentiation of K562 cells led to a time-dependent transcriptional induction of *Rac2* gene expression. Unlike early response genes such as *c-Jun*, *c-Fos*, and *TNF α* (Yeh et al., 1992; Karin et al., 1997; Sullivan et al., 2007) that exhibit an induction of transcription minutes after PMA stimulation, *Rac2* gene expression is induced after a lag of 6-12 hrs as a secondary response to PMA treatment. Similar to *Rac2*, transcription of inflammatory mediators such as IL-6 and IL-12 is also delayed following cytokine stimulation (Sullivan et al., 2007). Early and late response genes rely on similar transcription factors, such as NF- κ B, AP1, and C/EBP, to activate transcription. But unlike the promoters of early response genes that are immediately accessible to transcription factors, the chromatin structure of secondary response genes must be modified to allow transcription factor binding (Saccani et al., 2001).

A 135 bp regulatory element within the -4.2 to -4 kb *Rac2* promoter region is necessary and sufficient for transcriptional induction of the *Rac2* gene following PMA stimulation. This regulatory region contains three consensus AP1 binding sites to which AP1 binds both *in vitro* and *in vivo*. Over-expression of the c-Jun/c-Fos AP1 heterodimer is sufficient to induce *Rac2* promoter activity, and both c-Jun and c-Fos are detected binding to the *Rac2* promoter. However, these studies do not rule out the possibility that additional factors that can comprise AP1 binding activity may also participate in *Rac2* expression. The Oligo 4 and Oligo 7 EMSA probes produce multiple retarded bands when mixed with PMA-stimulated nuclear extract, suggesting that more than one AP1 protein complex may bind to the AP1 consensus site (Fig 4). Though c-Jun and c-Fos are the prototype AP1 components, AP1 is composed of many different combinations of c-Jun, JunB, JunD, c-Fos, Fos B, Fra1, Fra2, ATF2, ATFa and ATF3. Multiple AP1 subunits can be expressed at the same time and compete for binding to the AP1 consensus site (Cohen et al., 1989). The competition between these subunits for binding to AP1 consensus site can also influence their trans-activation activity (Hess et al., 2004).

Site-directed mutagenesis revealed that each of the AP1 binding sites contributes to the PMA-responsiveness of the *Rac2* promoter. Recruitment of AP1 to all three sites may facilitate DNA bending (Kerppola, 1998), thereby augmenting recruitment of RNA pol II to the proximal promoter. Similar AP1-mediated enrichment of RNA pol II increases transcription of the *ccl2* chemokine gene (Wolter et al., 2008). Close spacing of AP1 sites can favor formation of

a loop structure that brings an enhancer in close proximity to the transcription initiation complex (Ney et al., 1990; Chytil et al., 1998; Chinenov and Kerppola, 2001). Also, tandem AP1 sites within the human β -globin dominant control region function as an inducible enhancer in erythroid cells (Ney et al., 1990).

Despite an induction of *in vitro* AP1 DNA-binding activity within an hour of PMA stimulation, induction of the *Rac2* gene is delayed 6-12 hrs, consistent with a 5-9 hrs delay in the appearance of AP1 binding to the endogenous *Rac2* promoter. Furthermore, the expression of *Rac2* promoter/luciferase constructs, but not the endogenous *Rac2* gene, is induced upon over-expression of AP1 in the absence of PMA stimulation. Together, these data suggest that chromatin structure plays an important role in controlling expression of the endogenous *Rac2* gene by regulating access of AP1 to the 135 bp *Rac2* regulatory region. Consistent with this model, the 135 bp *Rac2* regulatory region exhibits alterations of chromatin structure following PMA stimulation, including a decrease in histone H3K9 dimethylation, an increase in histone H3 acetylation, and increased sensitivity to MNase, that are characteristic of transcriptionally competent genes. However, treatment of cells with the histone deacetylase inhibitor trichostatin A was not sufficient to induce expression of the endogenous *Rac2* gene following over-expression of AP1 (data not shown).

Previous work by our laboratory implicated chromatin structure in the repression of *Rac2* gene expression in non-hematopoietic cells. Sequences flanking both the 3'- and 5'-ends of the *Rac2* gene contain abundant cytosine methylation in non-expressing cell lines and tissues, but these sequences are specifically demethylated in hematopoietic cells (Ladd et al., 2004). Furthermore, demethylation of the *Rac2* locus following aza-cytosine treatment is sufficient to induce expression of the endogenous *Rac2* gene in the non-hematopoietic HEK-293 cell line (Ladd et al., 2004). In contrast, the studies described here implicate histone modifications as playing a critical role in the modulation of *Rac2* expression levels during terminal myeloid cell differentiation. Thus, in aggregate these studies reveal that regulation of cytosine methylation and histone modifications control distinct aspects of *Rac2* gene regulation, and illustrate the complex interplay of both regulated chromatin structure and sequence-specific transcription factors that direct lineage-and developmentally-restricted *Rac2* expression.

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Abbreviations used in this paper

ChIP	chromatin immunoprecipitation assay
CMV	cytomegalovirus
DMSO	dimethyl sulfoxide
EMSA	electrophoretic mobility shift assay
MNase	micrococcal nuclease
PCR	polymerase chain reaction
PMA	phorbol-12-myristate-13-acetate
PDD	phorbol-12,-13-didecanoate

Fig. 1. Rac2 mRNA levels are induced upon PMA stimulation and megakaryocytic differentiation of K562 cells

A) Light microscopy analysis of K562 cells grown for three days in medium containing DMSO, 100 nM PMA, or 100 nM PDD. Magnification 20X.

B) K562 cells were treated as in (A) for 24 hrs and surface staining of human CD41 was performed using PE-conjugated CD41 antibodies and analyzed by flow cytometry. Mouse IgG2A was used as an isotype control.

C) Quantitative real-time PCR analysis was performed to quantify Rac2 mRNA levels in K562 cells treated with DMSO, 100 nM PMA, or PDD for 24 hrs. ΔC_t (threshold cycle) values of Rac2 mRNA were normalized to ΔC_t values of Actin mRNA from the same cDNA

preparations. Normalized Rac2 transcript levels in K562 cells treated with PMA and PDD were then calibrated against the Rac2 transcript level in cells treated with DMSO. The data represent a summary of three independent experiments. Error bars indicate the standard error, and the asterisk indicates a p value of <0.05 compared to the DMSO control.

Fig. 2. Transcription of the *Rac2* gene increases upon PMA stimulation

A) *Rac2* mRNA levels from K562 cells stimulated with 100 nM PMA for 0, 6, 12, or 24 hrs were analyzed by quantitative real-time PCR analysis. ΔC_t values of *Rac2* mRNA were normalized to ΔC_t values of *Actin* mRNA from the same cDNA preparations. The data represent a summary of at least three independent experiments. Error bars indicate the standard error, and asterisks indicate a p value of <0.05 compared to the 0 hr time point.

B) Chromatin was isolated from K562 cells treated with PMA for various time periods and immunoprecipitated using anti-RNA polymerase II (RNAP II) antibody or normal rabbit serum (IgG). PCR analysis was performed on genomic DNA extracted from input samples (1% of the diluted lysate) and the immunoprecipitates using amplicons from a 200 bp intragenic region

(+4 kb) of the *Rac2* gene locus and a 200 bp intragenic region (+4 kb) of the *Actin* gene locus. The figure shown is representative of at least three independent experiments.

Fig. 3. The 4.5 kb proximal *Rac2* promoter contains PMA responsive cis-elements

A) The pGL3-luciferase vector or pGL3-luciferase vector containing the 4.5 kb proximal *Rac2* promoter, EF1 α promoter, or SV40 promoter were transiently transfected into K562 cells. After transfection the cells were divided into two samples, and one was treated with 100 nM PMA and the other with DMSO for 24 hrs. The cells were harvested and the relative luciferase activity of the PMA-treated samples compared to the DMSO controls was calculated and represented as fold-induction of luciferase units. Results represent normalized mean $\pm$ standard error and are representative of at least six independent experiments using multiple plasmid preparations, performed in triplicate. *p* values were determined by a standard *t* test, and asterisks indicate *p* values of <0.05 relative to the empty pGL3-luciferase vector.

B) Various lengths of 5' - deletion constructs of the 4.5 kb human *Rac2* promoter were subcloned into the pGL3-Basic reporter gene vector. All constructs extend to +134 bp of the *Rac2* gene. Arrows indicate the transcription start site at the +1 position. Plasmids were transiently transfected into K562 cells and analyzed as in (A). The -34 to +134 bp *Rac2* gene region is denoted as "core *Rac2*". Results represent normalized mean $\pm$ standard error and are representative of at least six independent experiments using multiple plasmid preparations performed in triplicate, and asterisks indicate *p* values of <0.05 .

C) Transient transfection of K562 cells was performed using the pGL3-luciferase vector containing the 135 bp *Rac2* regulatory region inserted upstream of the core (-34 to +134 bp) *Rac2* promoter (135 bp + core *Rac2*), the core *Rac2* promoter/luciferase construct, or the parental pGL3-luciferase vector, and cells were treated with PMA or DMSO as in (A). The fold-induction of luciferase activity of the PMA-treated samples relative to DMSO controls is presented. Results represent normalized mean $\pm$ standard error and are representative of at least six independent experiments with multiple plasmid preparations performed in triplicate, and the asterisk indicates a *p* value of <0.05 compared to the core *Rac2*/luciferase construct.

Fig. 4. AP1 binds to three distinct regions within the 135 bp *Rac2* gene regulatory region
A) Schematic representation of the 135 bp *Rac2* regulatory region. The positions of seven overlapping oligo probes are indicated. Three putative AP1 binding sites are indicated.
B) EMSA was performed using seven overlapping oligo probes (Oligo 1-7). Radiolabeled probes were incubated with 5 µg of nuclear extract isolated from K562 cells treated for 24 hrs with DMSO or 100 nM PMA. DNA-protein complexes that are induced by PMA treatment are indicated by arrows for Oligo 1, 4, and 7 probes.
C) Oligo 1, 4, or 7 probes were incubated with 5 µg of nuclear extract isolated from K562 cells treated for 24 hrs with DMSO or 100 nM PMA. Competition experiments were performed by adding to the binding reaction a 70-fold excess of non-radiolabeled Oligo 1, Oligo 4, Oligo 7,

AP1 control oligo, or AP1 mutant oligo with PMA-treated samples. Nuclear extract was isolated from K562 cells treated with PMA for 24 hrs. AP1 probe denotes AP1 control oligonucleotide probe incubated with 5 μ g of nuclear extract isolated from K562 cells treated with 100 nM PMA.

D) Supershift analysis was performed by mixing radiolabeled Oligos 1, 4, or 7 probes with 5 μ g of nuclear extract isolated from K562 cells treated with 100 nM PMA for 24 hrs along with antibodies directed against c-Jun or c-Fos. Arrows indicate shifted complexes, and SS indicates supershifts.

Fig. 5. AP1 binds to the 135 bp *Rac2* regulatory region *in vivo*

Chromatin was prepared from K562 cells treated for 24 hrs with DMSO or 100 nM PMA. ChIP assays were performed using antibodies directed against c-Jun, c-Fos, or normal rabbit serum. The figure presents the results of PCR analysis of genomic DNA extracted from input samples and the immunoprecipitates using amplicons from the 135 bp *Rac2* regulatory region and a 175 bp intragenic region (+15 kb) of the *Rac2* locus. The figure shown is representative of at least three independent experiments.

Fig. 6. Functional activity of AP1 binding sites within the 135 bp *Rac2* regulatory region and trans-activation of the *Rac2* gene promoter by AP1

A) Single (Mut1, Mut4, or Mut7) or triple (Mut 147) mutations of AP1 binding sites were introduced into the 135 bp *Rac2* regulatory region and subcloned upstream of the core (-30 to +134 bp) *Rac2* promoter/luciferase vector. After transient transfection, K562 cells were divided into two samples and one was treated for 24 hrs with 100 nM PMA and other with DMSO. The cells were harvested and the relative luciferase activity of the PMA-treated samples compared to the DMSO controls was calculated and represented as fold-induction of luciferase units. Results represent normalized mean $\pm$ standard error and are representative of at least six independent experiments performed in triplicate. *p* values were determined by a standard *t* test, and asterisks indicate a *p* value of <0.05 compared to the wild type 135 bp + core *Rac2*/luciferase construct.

B) K562 cells were co-transfected with the 135 bp + core *Rac2*/luciferase plasmid and various combinations of AP1 expression vectors. A CMV- β -galactosidase vector was also included in the transfection to serve as an internal control for transfection efficiency. Twenty four hours after transfection the cells were harvested, and luciferase and β -galactosidase activities were determined. After normalization with β -galactosidase activity, the difference in luciferase activity from cells transfected with c-Jun and/or c-Fos expression vectors compared to the empty vector was calculated and represented as relative luciferase units. Results represent normalized mean $\pm$ standard error and are representative of at least three independent experiments using multiple plasmid preparations performed in duplicate, and the asterisk indicates a *P* value of <0.05 .

C) Quantitative real-time PCR analysis was performed to quantify *Rac2* mRNA levels in K562 cells transfected with c-Jun and/or c-Fos expression vectors for 24 hrs. ΔC_t values of *Rac2* mRNA were normalized to ΔC_t values of Actin mRNA from the same cDNA preparations. The *Rac2* mRNA levels for samples transfected with empty expression vector was arbitrarily set as 1. The data shown are representative of two separate experiments performed in duplicate.

Fig. 7. Kinetics of AP1 binding to the 135 bp *Rac2* regulatory region following PMA induction
EMSA was performed using Oligo 1, 4, or 7 probes. Radiolabeled probes were incubated with 5 μ g of nuclear extract isolated from K562 cells treated for 0, 1, 3, or 6 hrs with 100 nM PMA. DNA-protein complexes that are induced by PMA treatment are indicated by arrows.

Fig. 8. Chromatin remodeling of the 135 bp *Rac2* gene promoter region upon PMA stimulation of K562 cells

A) Chromatin was isolated from K562 cells treated with DMSO or 100 nM PMA for 24 hrs and subjected to ChIP analysis with antibodies directed against acetylated histone H3, dimethylated histone H3K9, or normal rabbit serum. The figure shows the analysis of genomic DNA extracted from input samples and immunoprecipitates using amplicons from the 135 bp *Rac2* regulatory region. The data are representative of two independent experiments.

B) Intact nuclei were isolated from K562 cells treated with DMSO or 100 nM PMA for 24 h and subjected to treatment with 100 U of MNase for 30 min. Genomic DNA was extracted and the 135 bp *Rac2* regulatory region was PCR amplified to assess chromatin accessibility. The data are representative of three independent experiments.

C) Chromatin was prepared from K562 cells treated 100 nM PMA for 0, 5, 9, or 12 hrs and subjected to ChIP analysis with antibodies directed against c-Jun, acetylated histone H3, dimethylated histone H3K9, or normal rabbit serum. The figure shows the results of PCR analysis of genomic DNA extracted from input samples and immunoprecipitates using amplicons from the 135 bp *Rac2* regulatory region. The data are representative of three independent experiments.

D) Intact nuclei were isolated from K562 cells treated with 100 nM PMA for 0, 5, 9, or 24 hrs and subjected to treatment with 100 U of MNase. Genomic DNA was extracted and probed for the 135 bp *Rac2* regulatory region was amplified by PCR. The data are representative of two independent experiments. The graph represents the ratio of the intensity of the PCR product from samples digested with MNase to the intensity of the PCR product from undigested samples.