Proteomic Analysis Yields an Unexpected Trans-Acting Point in Control of the Human Sympathochromaffin Phenotype

Stéphane Chiron, PhD; Zhiyun Wei, BS; Yuqing Chen, MD, PhD; Kuixing Zhang, MD, PhD; Gen Wen, MD, PhD; Wolfgang H. Fischer, PhD; Sushil K. Mahata, PhD; Daniel T. O’Connor, MD

Background—The secretory protein chromogranin A (CHGA) plays a necessary role in formation of catecholamine storage vesicles and gives rise to a catecholamine release–inhibitory fragment. Because genetic variation in the proximal human CHGA promoter predicts autonomic function and blood pressure, we explored how a common genetic variant alters transcription of the gene.

Methods and Results—Bioinformatic analysis suggested that the common G→462A promoter variant (rs9658634) may disrupt as many as 3 transcriptional control motifs: LEF1, COUP-TF, and PPARγ-RXRα. During electrophoretic mobility shifts, chromaffin cell nuclear proteins bound specifically to the A (though not G) allele of CHGA promoter G→462A. On oligonucleotide affinity chromatography followed by electrospray ionization followed by 2-dimensional (tandem) mass spectrometry analysis of A allele eluates, the transcription factor LEF1 (lymphoid enhancer-binding factor-1) was identified. Interaction of LEF1 with the A allele at G→462A was confirmed by supershift. On cotransfection, LEF1 discriminated between the allelic variants, especially in chromaffin cells. Allele specificity of trans-activation by LEF1 was transferable to an isolated G→462A element fused to a heterologous (SV40) promoter. Because β-catenin (CTNNB1) can heterodimerize with LEF1, we tested the effect of cotransfection of this factor and again found A allele–specific perturbation of CHGA transcription.

Conclusions—Common genetic variation within the human CHGA promoter alters the interaction of specific factors in trans with the promoter, with LEF1 identified by proteomic analysis and confirmed by supershift. Coexpression experiments show functional effects of LEF1 and CTNNB1 on CHGA promoter. The findings document a novel role for components of the immune and WNT pathways in control of human sympathochromaffin phenotypes. (Circ Cardiovasc Genet. 2011;4:437-445.)

Key Words: chromaffin • chromogranin • catecholamine • transcription

Chromogranin A (CHGA) is a 48-kDa acidic protein directed to the regulated secretory pathway in neuroendocrine cells and a major constituent of regulated secretory vesicles, also designated dense core granules (DCGs). DCGs serve as a storage compartment for catecholamines in postganglionic sympathetic neurons and adrenal medullary chromaffin cells, from which all granule core contents are coreleased on stimulation by exocytosis. Studies have shown that CHGA may be both necessary and sufficient for the formation of a regulated secretory pathway. In addition to this important role in DCG formation, CHGA is a prohormone, coreleased with catecholamines from the DCGs on stimulation. On processing at dibasic sites, the prohormone CHGA gives rise to biologically active peptides such as the inhibitor of catecholamine release catenatatin, the vasodilator vasostatin, or the dysglycemic peptide pancreastatin.

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Hypertension displays substantial heritability in family studies, but the genetic contributions to control of blood pressure are still poorly understood. On the basis of observations in humans and rodents, we proposed that CHGA formation and secretion may constitute “intermediate” (early or pathogenic) phenotypes for later development of hypertension. For instance, CHGA is overexpressed in adrenal medulla of rodent hypertension, both spontaneous and acquired. Moreover, ablation of the CHGA gene in mice leads to substantial hypertension. Finally, CHGA plasma levels parallel catecholamine release in human populations, and phenotypic links between CHGA and human hypertension are reported.

A detailed study of naturally occurring genetic variations at the human CHGA locus revealed 20 common single-nucleotide...
polymorphisms (SNPs), including 8 variants in the 1.2-kbp proximal promoter. From the 8 promoter haplotypes inferred, 2 showed substantially different transcriptional activities in reporter gene experiments. These 2 haplotypes span 3 variants (T→1014C→G→988T→G→462A) in linkage disequilibrium (LD) that govern CHGA expression. Further analysis of these 3 SNPs revealed a major contribution of the SNP G→462A (rs9658634) for modification of transcriptional activity of the isolated CHGA promoter. In addition, variations in this promoter region predict blood pressure increase after environmental stress as well as basal blood pressure; initial analysis of the −462 position suggested involvement of the transcription factor COUP-TF.

In the present study, we revisited the cellular mechanisms that may occur at the −462 position of the CHGA promoter, using an unbiased, hypothesis-free, proteomic approach. We find not only compelling new computational evidence of differing protein-binding specificity between the wild-type and the variant promoter regions but also biochemical as well as functional evidence for binding of lymphoid enhancer factor-1 (LEF1) at this molecular switch region of the CHGA promoter.

Materials and Methods

Cell Culture
The rat adrenomedullary chromaffin cell line PC12 was grown in high-glucose Dulbecco’s modified Eagle’s medium with penicillin G (100 U/mL) and streptomycin sulfate (100 mg/mL), supplemented with 10% horse serum and 5% fetal bovine serum.

Promoter/Luciferase Reporter Plasmids
Human CHGA promoter/reporter plasmids were constructed essentially as previously described. Haplo-type-I promoter fragment corresponding to CHGA −1142/+54 bp (with respect to the cap site) was amplified from genomic DNA of known homozygotes and cloned between the site Kpn I and Xho I in the pGL3-Basic vector (Promega Inc, Madison, WI). The rs9658634 SNP was reintroduced by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA). For modification of the pGL3-Promoter plasmid, the oligonucleotides CACTCAGACAGGGGATC were annealed with their complements and cloned into pCMV-driven transcription factor complex. These 2 haplotypes span 3 variants (T→1014C→G→988T→G→462A) in linkage disequilibrium (LD) that govern CHGA expression. Further analysis of these 3 SNPs revealed a major contribution of the SNP G→462A (rs9658634) for modification of transcriptional activity of the isolated CHGA promoter.

Transfection and Reporter Assay
The PC12 cell line was transfected at 50% to 60% confluence by the liposome method (TransfectIN, Bio-Rad) according to the manufacturer; with 500 ng reporter plasmid and 2.75 μL of TransfectIN per well of a 24-well plate. For Remilla luciferase control, 50 ng of plasmid pRL-TK (Promega Inc) was cotransfected. For transactivation experiments, 50 ng of plasmid in pCMV vector/transcription factor or pcDNA3.1 (control) was cotransfected. Cotransfected pCMV-driven trans-activator plasmid cDNAs were human LEF1 (Open Biosystems, Huntsville, AL) or Xenopus CTNNB1 (Open Biosystems). Cells were lysed 18 to 24 hours after transfection in 100 mmol/L KOH, pH 7.8, 1 mmol/L DTT, 0.1% Triton X-100. For dual-luciferase measurements, Stop & Glo (Promega Inc) as well as homemade buffers were used. Each experimental condition was repeated a minimum of 4 times (replicates).

EMSA and Supershift
PC12 nuclear extracts were obtained with the use of a nuclear extraction kit (Cayman). The oligonucleotide probes (see promoter/luciferase reporter plasmids, previous section) were biotin-labeled using the Biotin 3‘ End DNA Labeling Kit (Pierce) and annealed. Nuclear extract–binding reactions were performed at room temperature for 20 minutes using 7 μg of nuclear extract and 20 fmol of biotin-labeled oligonucleotide in 15 μL final volume, using a LightShift chemiluminescent EMSA kit (Pierce). Protein–oligonucleotide probe complexes were resolved using native 5% polyacrylamide gels and transferred onto nitrocellulose membranes. The biotin-tagged oligonucleotides were detected with the use of a Chemiluminescent Nucleic Acid Detection Module (Pierce), in which a streptavidin–horseradish peroxidase conjugate was hybridized to the membrane, washed, and visualized by luminol chemiluminescence.

Purification of Sequence-Specific DNA-Binding Proteins by Affinity Chromatography
Nuclear extracts were prepared from PC12 cells as described for EMSA. Affinity purification was performed as described, with modifications. 3‘-TEG–biotinylated versions of the forward oligonucleotide probes used for EMSA were annealed to their unlabeled complements. The ds-oligonucleotides (25 pmol) were captured on 150 μg of M280 streptavidin magnetic beads for 30 minutes (Dynal Biotech, Oslo, Norway; Invitrogen). All purification procedures were carried out at 4°C. Crude nuclear extracts (~115 μg) were mixed with 150 μg of salmon sperm DNA in a 10 mmol/L Tris, pH 7.5, 50 mmol/L KCl, 1 mmol/L DTT buffer and incubated 30 minutes with streptavidin beads to clear extracts from proteins binding the streptavidin or the beads. Precleared extract was then incubated with beads coated with G or A allele oligonucleotides. Beads were washed 3 times with 100 mmol/L Tris, pH 7.5, 100 mmol/L KCl buffer. The beads were eluted in 50 μL of Laemmli buffer. Elutions were separated on SDS-PAGE and were Coomassie blue–stained (SimplyBlue SafeStain, Invitrogen). Coomassie blue–stained protein bands were excised and in-gel–digested with trypsin and analyzed by liquid chromatography (LC) electrospray ionization mass spectrometry (MS) as described. Briefly, samples were loaded onto a capillary column with an integrated spray tip (75-μm ID, 10-μm tip, New Objective, Woburn, MA), which was packed in-house with C18 reversed-phase material (Zorbax SB-C18, 5-μm particle size, Agilent, Santa Clara, CA) to a length of 10 cm. The reversed-phase elution was achieved by a linear gradient of 0% to 60% acetonitrile in 0.1% formic acid within 60 minutes at a flow rate of 300 nL/min. The eluate was introduced into a Thermo LTQ-Orbitrap mass spectrometer (ThermoFisher, Waltham, MA) through a nanospray source. Mass spectrometric analysis was conducted by recording precursor ion scans at a resolution of 60 000 in the Orbitrap Fourier-transform analyzer followed by collision-induced dissociation MS/MS scans of the top 5 ions in the linear ion trap (cycle time, approximately 1 second). An active exclusion window of 90 seconds was used. Data were analyzed on a Sorcerer Solo system running Sorcerer-Sequest (rev11) against the IPF rat data base (v3.61, RAT, 39876 entries) and by using the Mascot algorithm (V. 27 rev.11, Matrix Science, London, UK). Scaffold (version Scaffold_2_06_02, Proteome Software Inc, Portland, OR) was used to validate MS/MS, based peptide and protein identifications. Scaffold (version 3.61, RAT, 39876 entries) and by using the Mascot algorithm. 3.61 position suggested involvement of the transcription factor COUP-TF.

In supershift assays, 1 μL of antibody was added before migration and incubated 20 minutes. The antibodies were from Santa Cruz Biotechnology: PPARγ, sc-2727X; COUP-TF, sc-30180X; and LEF1, sc-8591X. Details of the LEF1 motif sequence specificity, by position weight matrix derived from experimental data, are available at the Chip-Mapper interface for the Transfac data base.


Purification of Sequence-Specific DNA-Binding Proteins
Nuclear extracts were prepared from PC12 cells as described for EMSA. Affinity purification was performed as described, with modifications. 3‘-TEG–biotinylated versions of the forward oligonucleotide probes used for EMSA were annealed to their unlabeled complements. The ds-oligonucleotides (25 pmol) were captured on 150 μg of M280 streptavidin magnetic beads for 30 minutes (Dynal Biotech, Oslo, Norway; Invitrogen). All purification procedures were carried out at 4°C. Crude nuclear extracts (~115 μg) were mixed with 150 μg of salmon sperm DNA in a 10 mmol/L Tris, pH 7.5, 50 mmol/L KCl, 1 mmol/L DTT buffer and incubated 30 minutes with streptavidin beads to clear extracts from proteins binding the streptavidin or the beads. Precleared extract was then incubated with beads coated with G or A allele oligonucleotides. Beads were washed 3 times with 100 mmol/L Tris, pH 7.5, 100 mmol/L KCl buffer. The beads were eluted in 50 μL of Laemmli buffer. Elutions were separated on SDS-PAGE and were Coomassie blue–stained (SimplyBlue SafeStain, Invitrogen). Coomassie blue–stained protein bands were excised and in-gel–digested with trypsin and analyzed by liquid chromatography (LC) electrospray ionization mass spectrometry (MS) as described. Briefly, samples were loaded onto a capillary column with an integrated spray tip (75-μm ID, 10-μm tip, New Objective, Woburn, MA), which was packed in-house with C18 reversed-phase material (Zorbax SB-C18, 5-μm particle size, Agilent, Santa Clara, CA) to a length of 10 cm. The reversed-phase elution was achieved by a linear gradient of 0% to 60% acetonitrile in 0.1% formic acid within 60 minutes at a flow rate of 300 nL/min. The eluate was introduced into a Thermo LTQ-Orbitrap mass spectrometer (ThermoFisher, Waltham, MA) through a nanospray source. Mass spectrometric analysis was conducted by recording precursor ion scans at a resolution of 60 000 in the Orbitrap Fourier-transform analyzer followed by collision-induced dissociation MS/MS scans of the top 5 ions in the linear ion trap (cycle time, approximately 1 second). An active exclusion window of 90 seconds was used. Data were analyzed on a Sorcerer Solo system running Sorcerer-Sequest (rev11) against the IPF rat data base (v3.61, RAT, 39876 entries) and by using the Mascot algorithm (V. 27 rev.11, Matrix Science, London, UK). Scaffold (version Scaffold_2_06_02, Proteome Software Inc, Portland, OR) was used to validate MS/MS, based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at >99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by Protein Prophet. Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principle of parsimony.
**Chromatin Immunoprecipitation**

ChIP was accomplished by modification of procedures previously described by us.\(^5\) PC12 chromaffin cells were transfected with particular CHGA promoter haplotype/reporters to obtain G versus A alleles for the G/462A variant. ChIP assays were carried out using the Imprint ChIP kit (CHP1; Sigma, St Louis, MO). Cells (in transfected 10-cm plates) were cross-linked in 1% formaldehyde for 10 minutes at room temperature and washed with ice-cold PBS, then resuspended in nuclear preparation buffer. Chromatin was sonicated to achieve internucleosomal cleavage (Branson Sonifier) until DNA was fragmented to 500 to 1000 bp size. After 10-minute centrifugation, samples were incubated with specific or control antibodies preadsorbed to polystyrene wells at room temperature for 1.5 hours with rotation. Characteristics of the specific antibody were goat polyclonal anti-LEF1, SCBT sc-8591X. Control antibodies were from the Sigma ChIP kit: preimmune normal mouse IgG (as a negative control) and anti-RNA polymerase II (as a positive control). The adsorbed immune complex washed 6 to 7 times and eluted by "DNA release buffer" including proteinase K digestion at 65°C for 15 minutes; cross-links were then reversed with "reversing solution" with heating in a 65°C for 1.5 hours. The DNA was subsequently extracted and purified with GenElute Binding Column G (Sigma). Immunoprecipitated nucleosomal DNA samples were analyzed by PCR, using primers forming a 152-bp amplicon that bracketed the G/462A (sense: 5'-AGAGAGAAGCCTCACTCAGACAG-3', antisense: 5'-CACCCCGTGCTATTTCCTA-3') site in the human CHGA promoter. Extracted DNA from the chromatin fractions before antibody adsorption/elution was used as a positive control ("input DNA"). To ensure that the PCR amplification was in the linear range, reactions with different amounts of input DNA samples were carried out for various (typically 15 to 30) cycle numbers; a linear range of amplification typically occurred at 25 cycles. After amplification, PCR products were separated on 1.5% agarose TBE gels. In this reporter system, the transfected plasmid is incorporated into the chromatin fraction of the cell.\(^5\)

**Statistics**

Analyses were performed in SPSS. Results are expressed as mean±1 SEM. Cell culture experiments were typically done with 4 replicates per condition. Parametric statistics (\(t\) test) were used to evaluate differences between experimental conditions because descriptive statistics were consistent with approximately normal distributions of the data points (skewness from −0.8 to +0.8).

**Results**

**Bioinformatic Analysis Indicates 5 Putative Binding Motifs in the CHGA Promoter Region Encompassing the G−462A SNP**

Because CHGA proximal promoter SNPs in the region T−1014C→G−988T→G−462A display peak predictions of human traits, including CHGA plasma level\(^2\) and blood

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**Table A**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Model</th>
<th>Strand</th>
<th>Start</th>
<th>End</th>
<th>Score</th>
<th>E-val</th>
<th>Alignment (model, match)</th>
<th>(\Delta) Score A-G</th>
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<td>6.1</td>
<td><em>-&gt;cttctattgag.t&lt;-</em></td>
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<tr>
<td>PPARG-RXR</td>
<td>M0065</td>
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<td>12</td>
<td>28</td>
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<td>1.4</td>
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<tr>
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<tr>
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<td>23</td>
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<tr>
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<td>3.3</td>
<td>6.6</td>
<td><em>-&gt;tacctttgcctcc&lt;-</em></td>
<td>3.3</td>
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**Figure 1.** Identification of binding motifs spanning position G−462A in the human CHGA promoter. **A**, Putative factors binding the CHGA promoter at the G−462A SNP position. A region of 31-bp spanning the −462 SNP in the variant CHGA promoter was analyzed using the software Mapper (http://bio.chip.org/mapper) for putative binding motifs. Shown are the hits with a score >3, the associated model and its properties, and the motif alignments (modeled motifs are represented between *-><*, with partial matches shown by +). Vertical arrows indicate the position of the G−462A variant (in italics). On the minus strand, the same variant is C/T. **B**, Schematic representation of the putative binding motifs and factors. Only the plus strand (−479 to −449) of the region spanning the −462 SNP (bold) is represented, together with the putative motifs on the plus strand (+, above the sequence) or on the reverse complement (−, below the sequence). **C**, LEF-1 homology match at human CHGA promoter variant G−462A across primate species.

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pressure response to environmental stress,\textsuperscript{20} and the SNP G\textsuperscript{462A} within this region was predominant in reporter gene experiments.\textsuperscript{20,21} We decided to search systematically for transcription-binding sites around the G\textsuperscript{462A} position, using contemporary bioinformatic and proteomic approaches. Bioinformatic analysis of a 31-bp sequence surrounding the SNP (from \(-479\) to \(-449\); highly conserved across primates) revealed several degenerate potential binding motifs in the JASPAR and TRANSFAC data bases (Figure 1A).

As expected by Chen et al.,\textsuperscript{20} 1 of the first 5 hits for the variant (minor allele, \(-462A\)) sequence was a partial match for the previously published consensus motif of COUP-TF (MA0017). However, we also found 4 new motifs spanning \(-462A\), with even higher scores than the COUP-TF prediction (Figure 1A). The best match corresponds to a binding motif for LEF1 (M00978), with a near-perfect homology.\textsuperscript{23} The second hit is a partial match for the binding sequence of the complex PPAR\textgamma-RXR\alpha (MA0065), whereas the third hit involves a hepatic factor, HNF4-\textalpha (M00411). The fourth hit corresponds to a different reported binding motif for LEF1\textsuperscript{23} (M00745), on the reverse complement of the first hit. Of these 5 strongest hits, 3 involve the “plus” strand of the promoter, whereas the 2 others indicate binding on the “minus” strand (Figure 1B).

When performed on the wild-type (major allele, G\textsuperscript{-462}) sequence, the analysis failed to identify partial matches for the factors LEF1 (M00978), HNF4-\textalpha (M00411), or COUP-TF (MMA0017).

Therefore, our results from the bioinformatic analysis suggest a new set of proteins that putatively bind the G\textsuperscript{462A} region of the CHGA promoter and could further explain the differences of transcriptional activity reported for the 2 alleles.\textsuperscript{20,21}

**Electrophoretic Mobility Shift Assay: Nuclear Proteins Bind to the A Allele of CHGA Promoter G\textsuperscript{462A} But Not to the G Allele**

To probe molecular mechanisms underlying the transcriptional regulation of G\textsuperscript{462}A and clearly identify the factor responsible for differences in expression, we analyzed by electrophoretic mobility shift assay (EMSA) the binding specificity of rat pheochromocytoma (chromaffin) cell line (PC12) nuclear extracts to the 31-bp sequences (alleles G\textsuperscript{462} and \(-462A\), Figure 2A) used in the bioinformatic analysis (Figure 1B). As shown in Figure 2B, the biotinylated double-stranded oligonucleotides incubated without PC12 nuclear extracts are visualized at higher mobility during electrophoresis, indicating free/unbound DNA probes (Figure 2B, lanes 1 and 4). Incubation of the G allele with PC12 nuclear extracts before electrophoresis reveals a very low mobility band (lane 2) that is not displaced by incubation with the same allele as competitor (nonbiotinylated DNA, lane 3). Moreover, this very low mobility band appears as well in the nuclear extract—only (ie, no oligonucleotide probe) control (lane 8), indicating a nonspecific product contained in PC12 nuclear extracts and recognized by the EMSA avidin reagents. Although no additional band is seen after incubation of the G allele with PC12 nuclear extract, the A allele DNA fragment shows a second shifted band (lane 5, asterisk), which is displaced by addition of the nonbiotinylated A allele DNA fragment (lane 6), indicating specificity. On the contrary, preincubation with a nonbiotinylated G allele DNA fragment fails to compete for the binding (lane 7), further illustrating the binding specificity of PC12 nuclear proteins for the A allele at G\textsuperscript{462A}.

**Proteomic Analysis of Nuclear Proteins Binding the A Allele of CHGA Promoter G\textsuperscript{462A}: Identification of Transcription Factor LEF1**

We used oligonucleotide affinity chromatography to proceed in a hypothesis-free fashion toward identification of the trans-acting factor(s) by tandem mass spectrometry. The positive strand of G or A allele DNA fragments were designed with a 3'-TEG-Biotin tag, annealed with their complementary (minus) strands, and incubated with streptavidin-conjugated magnetic beads (Figure 3A). To minimize nonspecific binding of proteins to the streptavidin or the magnetic beads, PC12 nuclear extracts were precleared by incubation with streptavidin-conjugated magnetic beads alone before to the incubation with the magnetic beads bearing the allele-specific DNA fragments. To monitor the purification procedure, each fraction of the oligonucleotide affinity chromatography was analyzed by SDS-PAGE and Coomassie blue staining. The SDS-PAGE analysis of the elution fractions shown in Figure 3B reveals a high MW band (M\(_r\) \~120 kDa) purified with both the A and G allele DNA fragments, as well as a lower MW band (M\(_r\) \~64 kDa; arrow), purified only in presence of the A allele DNA. Eluates of the SDS-PAGE lanes were submitted for protein identification by trypsin-LC electrospray ionization followed by 2-dimensional (tandem) mass spectrometry (electrospray ionization-MS/MS) analysis.

LEF1 was identified (Figure 3C and 3D) in the A allele elution fraction (Mascot scores in online-only Data Supplement Table I), whereas we failed to detect that factor in the G allele elution. Of note, LEF1 typically migrates with an apparent MW around 60 kDa, close to the band we noted on elution from the
A allele (Figure 3B, arrow). Abundant cellular proteins without known DNA-binding activity (presumably nonspecific contaminants; list in online-only Data Supplement Table II) were not considered further. Several known ssDNA-binding proteins and proteins involved in transcriptional machinery were also identified (Figure 3C).

Confirmation of Binding of LEF1 to the A allele of CHGA Promoter: EMSA With Supershift

To confirm the binding of LEF1, we performed an EMSA supershift assay. Antibodies directed against COUP-TF, PPARγ, or LEF1, as well as a negative control antibody (directed against Pax6) were added to the preincubated oligonucleotides and PC12 nuclear extract, before EMSA. Whereas the control and the anti–COUP-TF and anti-PPARγ antibodies did not alter the migration pattern of the A allele DNA fragment, anti-LEF1 antibody induced a supershift of the A allele DNA fragment/protein complex (Figure 4A, lane 5). This result is consistent with the bioinformatic analysis identifying the LEF1 motif (Figure 1A and 4B). Not only is the LEF1 motif a perfect match with the variant −462A (as compared with more degenerate matches with the PPARγ and COUP-TF motifs), but the −462 SNP position is also one of the bases with the strongest specificity within the motif (Figure 4B).

Chromatin immunoprecipitation (ChIP) was also undertaken (see Materials and Methods section), with an antibody directed against LEF1; after immunoisolation of nucleosomes, polymerase chain reaction (PCR) with a 152-bp amplicon spanning G−462A detected LEF1 binding to the CHGA promoter on both alleles (G and A; data not shown). However, there is a second, nonpolymorphic LEF1 partial consensus match on the human CHGA promoter minus strand at position 530−519 (ACTTTGTTGT; Mapper score 6.1) (http://genome.ufl.edu/mapper). Because position 530−519 is within the internucleosomal DNA fragment size range typically obtained during sonication (500 to 1000 bp), we cannot rely on ChIP for specific detection of LEF1 binding at the G−462A motif.

LEF1 Functionally Modifies CHGA Promoter Activity by Discriminating Between the 2 Allelic Variants at G−462A

We attempted to transactivate the 2 alleles of the CHGA promoter with putative binding factors identified previously,
using plasmids wherein the human CHGA promoter (spanning −1142→+54 bp, or ≈1.2 kbp) was cloned into pGL3-Basic (Promega) to control expression of the Firefly luciferase gene. The 2 CHGA promoters used in this study are promoter Hap-1 (containing the G allele) and Hap-1/A (identical except for the A allele, created by site-directed mutagenesis). These plasmids were cotransfected into rat chromaffin cells (PC12 pheochromocytoma, Figure 5), along with a plasmid expressing human LEF1 under the control of the CMV promoter, or the plasmid pcDNA3.1 (empty CMV promoter vector) as a negative control. Coexpression of LEF1 resulted in a preferential decline in CHGA A allele promoter expression as compared with the G allele (P=0.029; Figure 5A, left).

Figure 4. Identification of the binding factor of the A allele by EMSA. A, Supershift experiment using the biotinylated A allele probe from Figure 2. The probe was sequentially incubated with PC12 nuclear extract and antibodies directed against PPARγ, COUP-TF, or LEF1 (Santa Cruz Biotechnology; see Materials and Methods section). An antibody directed against Pax6 was used as a negative control (sc-32766X). The protein:DNA complexes (asterisk) and the protein:DNA:antibody complexes (arrowheads) are indicated. B, G and A allele −462 region and recognition motifs of PPARγ-RXRα, COUP-TF, and LEF1/TCF1. Wide, thin, and dotted lines indicate, respectively, a perfect match with the base in the motif, a second choice base, and third or fourth choice base. The variable base is presented in bold. WebLogo profiles of consensus base preference are from Chip-Mapper (http://mapper.chip.org/).
Because LEF1 characteristically heterodimerizes with the WNT signal transduction pathway component CTNNB1 and thus constitutes an efferent limb of WNT pathway signaling, we tested whether CTNNB1 perturbed CHGA expression. Transfected CTNNB1 trans-activated the cotransfected human CHGA promoter in chromaffin cells (Figure 6), and the effect was greater for the A than the G allele at G–462A (A>G, P<0.001). When CTNNB1 and LEF1 were cotransfected, the A>G difference persisted (P=0.003).

Discussion

Overview

Sympathetic signaling and outflow are key components in the regulation of blood pressure. Associations of the sympathetic pathway with hypertension and “intermediate” phenotypes contributing to blood pressure control have been documented. Chromogranin A plays crucial roles in the sympathoadrenal system; because of its involvement in DCG formation, regulation of catecholamine release, and production of bioactive peptides, CHGA may represent a key candidate gene for the regulation of the autonomic contributions to blood pressure. Accumulating evidence gathered in different models now documents direct links between CHGA and blood pressure regulation.13–15,20 The plasma cestatin (CHGA catecholamine releases–inhibitory peptide) levels were reduced in subjects with established hypertension and their at-risk siblings. In addition, variations in the CHGA promoter were associated with plasma CHGA concentration and blood pressure.20,21 Although the SNP rs9658634 at position G–462A appears to be the most important functional variant in the promoter,19 the molecular mechanisms underlying its effects are incompletely understood.

In the present study, we combined an unbiased, hypothesis-free, proteomic screening approach, along with bioinformatic analyses and gene reporter assays, to identify the trans-acting factor(s) affecting the −462 region of the human CHGA promoter.

Cis and Trans Mechanisms at Human CHGA G–462A: Bioinformatic and Experimental Approaches

Bioinformatic analysis of a putative protein-binding site in the promoter region spanning the −462 SNP revealed partial binding motifs for the transcription factors LEF1, HNF4-α1, and COUP-TF and the heterodimeric complex PPARγ-RXRα. Whereas the putative binding of COUP-TF was already suggested, the 4 other binding motifs were novel. The partial motif for PPARγ-RXRα in the CHGA promoter was of importance because CHGA expression responded to retinoic acid and because PPARγ has been linked to multiple complex diseases and disorders, including not only metabolic disease but also hypertension. As previously reported, COUP-TF is a putative binding protein of this promoter region that increases expression in PC12 cells; this effect appears to be linked to the −462 region and showed selectivity between the 2 alleles. However, this transcription factor was not identified in the proteomic approach, and an antibody directed against COUP-TF failed to supershift DNA-protein complexes during EMSA. Our unbiased proteomic approach identified LEF1 as binding the SNP region.
Cotransfection of LEF1 acts differentially on the 2 alleles in gene reporter experiments in PC12 cells, and this effect is transferable to a heterologous (SV40) core promoter by the −462 region. Finally, an anti-LEF1 antibody demonstrated supershifting of DNA-protein complexes in EMSA, identifying LEF1 as a factor binding the promoter region at the minor allele (A). Taken together, our results suggest that the factor LEF1 binds specifically the minor (A) allele at G−462A, inducing selective repression of its activity.

**Role of Transcription Factor LEF1**

LEF1 was first identified as a T-cell–specific transcription factor (also named TCF1α) regulating of expression of the TCR gene.\(^{23,34}\) The LEF1 transcription factor is developmentally regulated and expressed in pre-B and T lymphocytes\(^ {35}\) and is a component of the WNT signaling pathway;\(^ {36}\) indeed, LEF1 bears a β-catenin (CTNNB1)-binding domain, whereby heterodimerization potentiates the transcriptional activity of LEF1.\(^ {37}\) However, LEF1 is also expressed in sympathochromaffin cell types, such as PC12 chromaffin cells\(^ {36}\) (our data) and human pheochromocytoma,\(^ {38}\) as well as the normal adrenal gland.\(^ {39}\) In the mouse adrenal gland, LEF1 expression follows a circadian rhythm,\(^ {40}\) and LEF1 expression in rat adrenal medulla increases after multiple rounds of immobilization stress.\(^ {41}\) Targeted ablation of the LEF1 locus in the mouse results in a pleiotropic spectrum of consequences, including alterations in some populations of neural crest–derived cells.\(^ {42}\) We showed that CTNNB1 expression augmented human CHGA promoter activity (Figure 6), with an A>G preference at G−462A, either alone (P<0.001) or in combination with LEF1 coexpression (P=0.003); thus, CTNNB1 may reverse the usual suppression of CHGA transcription by LEF1 (Figure 5), in CHGA allele-specific fashion. The results open the way to understanding a new effect of WNT/CTNNB1 signaling: control of the sympathochromaffin phenotype.

**Conclusions and Perspectives**

In the present study, we identified LEF1 as a polymorphism-dependent transcriptional repressor of the CHGA promoter, centering on the common functional variant G−462A. Given our results and the observations that the blood pressure circadian rhythm synchronizes with changes in LEF1 expression\(^ {40}\) and that experimental stress induces elevations in blood pressure and LEF1 expression,\(^ {40}\) it is plausible to postulate that LEF1 might have a role in catecholamine synthesis and/or secretion as well as DCG formation, perhaps in concert with the WNT/CTNNB1 pathway. Future experimentation is likely to reveal new roles of this transcription factor and its implications for human autonomic biochemistry, physiology, and disease.

**Sources of Funding**

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**Disclosures**

None.

**References**

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Chromogranin A plays a necessary role in formation of catecholamine storage vesicles and also gives rise to a catecholamine release-inhibitory fragment. Here we explored how a common genetic variant alters transcription of the gene. Common promoter variant G→462A disrupted a binding motif for transcription factor LEF1 (Lymphoid Enhancer-binding Factor-1), which was then identified by oligonucleotide affinity chromatography followed by mass spectrometry, and confirmed by electrophoresis. Exogenous LEF1 discriminated between the allelic variants in chromaffin cells, in cooperation with beta-catenin, a component of the WNT signaling pathway. The findings document a novel role the immune and WNT pathways in control of the sympathochromaffin phenotype.

**CLINICAL PERSPECTIVE**

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Proteomic Analysis Yields an Unexpected Trans-Acting Point in Control of the Human Sympathochromaffin Phenotype
Stéphane Chiron, Zhiyun Wei, Yuqing Chen, Kuixing Zhang, Gen Wen, Wolfgang H. Fischer, Sushil K. Mahata and Daniel T. O'Connor

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SUPPLEMENTAL MATERIAL.

Chiron et al., 2011.
Supplementary Tables/Legends:

**Supplementary Table 1:** Mascot score table of possible matches for the peptide of Mr: 1170.6357

**Supplementary Table 2:** Peptide identification by LC-MS/MS of the fraction containing LEF1. All the identified peptides (minus keratins) are presented with their putative assignments.
Supp Table 1: Mascot scores for Mr 1171.

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Suppl Table 2: All identified peptides.