Both Rare and Common Polymorphisms Contribute Functional Variation at *CHGA,* a Regulator of Catecholamine Physiology

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The chromogranin/secretogranin proteins are costored and coreleased with catecholamines from secretory vesicles in chromaffin cells and noradrenergic neurons. Chromogranin A (CHGA) regulates catecholamine storage and release through intracellular (vesiculogenic) and extracellular (catecholamine release–inhibitory) mechanisms. *CHGA* is a candidate gene for autonomic dysfunction syndromes, including intermediate phenotypes that contribute to human hypertension. Here, we show a surprising pattern of *CHGA* variants that alter the expression and function of this gene, both in vivo and in vitro. Functional variants include both common alleles that quantitatively alter gene expression and rare alleles that qualitatively change the encoded product to alter the signaling potency of CHGA-derived catecholamine release–inhibitory catestatin peptides.

Introduction

Chromogranin A (CHGA [MIM 118910]) is a central regulator of catecholamine vesicle storage and release that also plays key signaling roles in subsequent physiology (Taupenot et al. 2003). CHGA is targeted to the regulated secretory pathway (Taupenot et al. 2002), where it binds calcium and catecholamines (Videen et al. 1992). CHGA also binds the vesicle membrane, where it can influence the release of calcium from secretory granules to the cytosolic exocytotic machinery in secretory cells by modulating the inositol 1,4,5-trisphosphate receptor/Ca^{2+} channel (Yoo et al. 2002). CHGA is required for formation of catecholamine secretory vesicles in chromaffin-lineage PC12 cells and is sufficient to induce a regulated secretion system in nonsecretory cells (Kim et al. 2001). Proteolytic cleavage of CHGA at dibasic sites (Barbosa et al. 1991; Eskeland et al. 1996) produces, in secretory vesicles, several biologically active peptides, including catestatin (human $CHGA_{352-372}$ and bovine $CHGA_{344-364}$). Exocytosis of catecholamine vesicles is stimulated by cholinergic input. Catestatin inhibits subsequent catecholamine release by antagonizing nicotinic receptors, thus constituting an au-

tocrine/paracrine feedback loop (Mahata et al. 1997). The formation (Taylor et al. 2000; Jiang et al. 2001), inhibitory mechanism (Mahata et al. 1997, 1999, 2000; Taupenot et al. 2000), and likely structure (Tsigelny et al. 1998) of catestatin have been recently characterized. Other biologically active CHGA fragments include the vascular smooth-muscle relaxant vasostatin (human $CHGA_{1-76}$), the dysglycemic peptide pancreastatin (human $CHGA_{250-301}$) (Cadman et al. 2002), and the antimicrobial peptides prochromacin (human $CHGA_{79-439}$) and chromacin (human $CHGA_{176-197}$), thought to play a role in the neuroendocrine stress response to systemic infection (Tasiemski et al. 2002).

We have previously proposed CHGA expression and activity as intermediate phenotypes in human hypertension (O'Connor et al. 2000). Hypertension shows substantial heritability in family studies, but the precise genes controlling blood pressure variability in the population remain poorly understood (Miall and Oldham 1963; O'Connor et al. 2000; Timberlake et al. 2001). Intermediate phenotypes—simpler, often monogenic, traits associated with multifactorial disease—offer an enticing approach to the genetics of common disorders with complex inheritance. CHGA is a likely regulator of intermediate phenotypes that contribute to hypertension (O'Connor et al. 2002). CHGA is overexpressed by chromaffin cells in rodent models of both genetic (spontaneously hypertensive rat [Schober et al. 1989; O'Connor et al. 1999]) and acquired (renovascular [Takiyyuddin et al. 1993]) hypertension, and twin studies have demonstrated heritability of plasma CHGA concentration in humans (Takiyyuddin et al. 1995). Con-

versely, plasma concentration of the catestatin fragment is diminished both in established hypertension and in still-normotensive offspring at genetic risk of developing the disease (Kennedy et al. 1998; Mahata et al. 2000; O'Connor et al. 2002). This suggests that expression levels of *CHGA* and its peptides act in the pathogenesis of hypertension and that steady-state levels of different fragments may be uncoupled from each other. To identify genetic variants in *CHGA* that might alter its function, we resequenced all eight exons and adjacent intronic regions, ~1.2 kbp of 5′ promoter, and two intronic conserved noncoding regions from 180 ethnically diverse human subjects. Here, we report identification of both quantitative variation in expression level mediated by common promoter haplotypes and qualitative variation in catestatin peptide mediated by rare polymorphisms that alter its inhibitory potency.

Material and Methods

Subjects and Clinical Characterization

A series of 180 individuals was studied, selected to span a diverse range of ethnicities. Ethnicity was established by self-identification. None of the subjects had a history of renal failure. Subject characteristics are defined as in previous articles (e.g., O'Connor et al. 2002). Subjects were volunteers from urban southern California (San Diego), and each subject gave informed, written consent; the protocol was approved by the University of California San Diego institutional review board (see table A [online only]).

Molecular Genetics

Genomic DNA was prepared from leukocytes in EDTA-anticoagulated blood, through use of PureGene extraction columns (Gentra Biosystems), as described by Herrmann et al. (2000). Public draft human (Lander et al. 2001) and mouse (Waterston et al. 2002) genome sequence was obtained from the University of California Santa Cruz (UCSC) Genome Browser (UCSC Genome Bioinformatics Web site). Promoter positions were numbered with respect to the mRNA cap (transcriptional initiation) site. PCR primers were designed by Primer3 (Rozen and Skaletsky 2000; Primer3 Web site) to span each of the eight exons, as well as to include 50–100 bp of flanking intronic sequence. Target sequences were amplified by PCR from 16 ng genomic DNA in a final volume of 20 μl. Products were treated with exonuclease I and shrimp alkaline phosphatase to remove primers and dNTPs prior to cycle sequencing with BigDye terminators (Applied Biosystems). Sequence was determined on an ABI 3100 automated sequencer and analyzed using the Phred/Phrap/Consed suite of software to provide base quality scores (Ewing and Green 1998; Ew-

ing et al. 1998; Gordon et al. 1998). Polymorphism and heterozygosity were detected using Polyphred (Nickerson et al. 1997; Rieder et al. 1998) and were manually confirmed. A subset of these data was cross-validated manually through use of base calls from Applied Biosystems software and visual inspection of trace files to identify heterozygotes. Rare SNPs were confirmed by resequencing in multiple individuals and from the reverse direction.

Statistical Analysis

Haplotypes were estimated from unphased genotypes by use of the PHASE program (Stephens et al. 2001). Haplotype homozygosities were confirmed by visual inspection. Pairwise linkage disequilibrium (LD) between each common SNP was quantified as D' and Δ^2 by use of the GOLD software package (Abecasis and Cookson 2000). Neutrality tests, including Tajima's D, Fu and Li's D, and the H test were performed using phased haplotype data in DnaSP (Rozas and Rozas 1999). Similar results were also obtained with Arlequin (Schneider et al. 2000), MEGA2 (Kumar et al. 2001), and hand calculations of Tajima's D. We used the F_{ST} statistic as a relative measure of population differentiation and calculated F_{ST} for the *CHGA* promoter through use of the Arlequin package (Schneider et al. 2000). *CHGA* promoter haplotype networks were constructed in Arlequin, which computed a minimum spanning tree (MST) from the matrix of pairwise distances calculated between all pairs of haplotypes. Similar trees were also constructed using the neighbor-joining, parsimony, and maximum-likelihood methods. One-way post hoc ANOVA with a Bonferroni correction was performed using the SPSS software package to check the significance of the in vivo association study and in vitro haplotype-specific *CHGA* promoter activity.

Molecular Modeling

Models were based on native, linear, human catestatin (hCHGA$_{352-372}$) nuclear magnetic resonance (NMR) structure in the Protein Data Bank. Point mutants were aligned to the wild-type backbone template and then subjected to energy-minimization/molecular mechanics (Mahata et al. 1998; Tsigelny et al. 1998).

Assays (CHGA Peptide Radioimmunoassays in Human Plasma)

EDTA-anticoagulated plasma was frozen and stored at −70°C prior to assay. CHGA region-specific radioimmunoassays for CHGA$_{17-38}$ (hydrophobic disulfide loop), CHGA$_{116-437}$ (large fragment), CHGA$_{284-301}$ (pancreastatin), and CHGA$_{361-372}$ (catestatin) were based on synthetic peptides and polyclonal rabbit antisera, as described elsewhere (Stridsberg 2000). ^{125}I-radiolabeling of

each peptide was enabled by either an endogenous or adventitious (terminal) Tyr residue. Several of the CHGA region-specific radioimmunoassays have been described in detail elsewhere (Stridsberg 2000; Stridsberg et al. 1995, 2000).

Synthetic Peptides

Particular CHGA regions, including linear, human catestatin $CHGA_{352-372}$ (S_{352}SMKLSFRARAYGFRGPGP-QL$_{372}$) or its point mutants (Gly364Ser; Pro370Leu), were synthesized by the solid-phase method, with 9-fluorenylmethoxycarbonyl (Fmoc) protection chemistry, as described elsewhere (Mahata et al. 2000). Peptides were purified to >95% homogeneity by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on C-18 silica columns. Authenticity and purity of peptides were further verified by analytical chromatography (RP-HPLC) and electrospray-ionization or matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

Catecholamine Secretion (Effect of Human Catestatin or Its Variants)

Rat PC12 pheochromocytoma cells were grown at 37°C with 6% CO_2, in 10-cm dishes or six-well plates, in Dulbecco's minimum essential/high glucose medium supplemented with fetal bovine and horse serum and penicillin/streptomycin. Norepinephrine secretion from PC12 cells was monitored as described elsewhere (Mahata et al. 2000). In brief, cells were labeled for 3 h with 1 μCi ^{3}H-L-norepinephrine (71.7 Ci/mmol) (DuPont/NEN) and then were incubated for 30 min, with or without the secretagogue nicotine (60 μM), in the presence or absence of peptide antagonists (0.1–10 μM). Release medium and cell lysates were assayed for ^{3}H-L-norepinephrine by liquid scintillation counting. Net secretion is calculated as nicotine-stimulated release minus basal catecholamine release.

CHGA *Promoter Haplotype/Reporter Activity Assays*

Human *CHGA* promoter/reporter plasmids were constructed essentially as described by Rozansky et al. (1994) and Wu et al. (1994). Haplotype-specific promoter fragments corresponding to *CHGA* −1142/+54 bp were amplified from genomic DNA of known homozygotes (or heterozygotes for the two least common haplotypes), cloned into promoterless firefly luciferase reporter plasmid (pGL3-Basic [Promega]). Synthetic replacements were made by site-directed mutagenesis (QuickChange [Stratagene]). All promoter fragments were sequence verified before use. Promoter positions are numbered upstream (−) or downstream (+) of the cap site. Plasmids were purified on columns (Qiagen) prior to transfection. PC12 pheochromocytoma cells were transfected (at 50%–60% confluence, 1 d after 1:4 splitting) with 1 μg of supercoiled promoter haplotype-firefly luciferase reporter plasmid and 10 ng of the *Renilla* luciferase expression plasmid pRL-TK (Promega) as an internal control per well, by the liposome method (Superfect [Qiagen]). The firefly and *Renilla* luciferase activities in the cell lysates were measured 16–21 h after transfection, and the results were expressed as the ratio of firefly/*Renilla* luciferase activity (Stop & Glo [Promega]). Each experiment was repeated a minimum of three times.

Results

To identify genetic variants in *CHGA* that might alter its function, we resequenced all eight exons and adjacent intronic regions, the proximal promoter, and two intronic conserved noncoding regions (≥75% conserved between human and mouse) from 180 ethnically diverse human subjects collected as part of our ongoing phenotypic studies (fig. 1). We identified 53 SNPs and 2 single-base insertion/deletions in this 5,725-bp footprint. Of these, 20 SNPs were common (minor allele frequency ≥5%) in our initial sample. It is surprising that 8 of the common SNPs (and 13 total) occur within 1,175 bp in the proximal promoter. The estimated nucleotide diversity (π) (Tajima 1989) in the promoter region (0.0021) is two- to threefold higher than in other regions of *CHGA,* including introns (table 1), and is also several-fold higher than promoter regions of other genes we have resequenced from the same subjects (0.0007, 0.0001, and 0.0004 for similar-sized segments of *PYY, PMX2B,* and *DBH,* respectively, and 0.0008 for combined smaller segments of *NPY2R* and *PNMT*). This excess variation is even more extreme in samples with African and European ancestry, since Asian samples in our data show no increase in nucleotide diversity for the *CHGA* promoter. Among 17 coding-region polymorphisms, 11 encode amino acid substitutions; two of these, heterozygous Gly364Ser and Pro370Leu (positions in the mature CHGA protein), are relatively unusual (minor allele frequency 0.6%–3.1%) variants in the catestatin peptide that seem to influence its function in inhibiting vesicular catecholamine release (table B [online only]). We completely resequenced *CHGA* from two chimpanzees to determine the likely ancestral alleles at polymorphic sites and verified this ancestry for sites of interest with additional sequence from bonobo and gorilla.

To identify variants that are inherited together, we inferred haplotypes from the 20 most common SNPs in *CHGA,* through use of PHASE (Stephens et al. 2001). We identified eight major haplotypes, accounting for 74.7% of chromosomes examined (fig. 1). These eight haplotypes explicitly include the common variation in coding and likely regulatory regions and serve as surrogates (by LD) for any unexamined regions that might
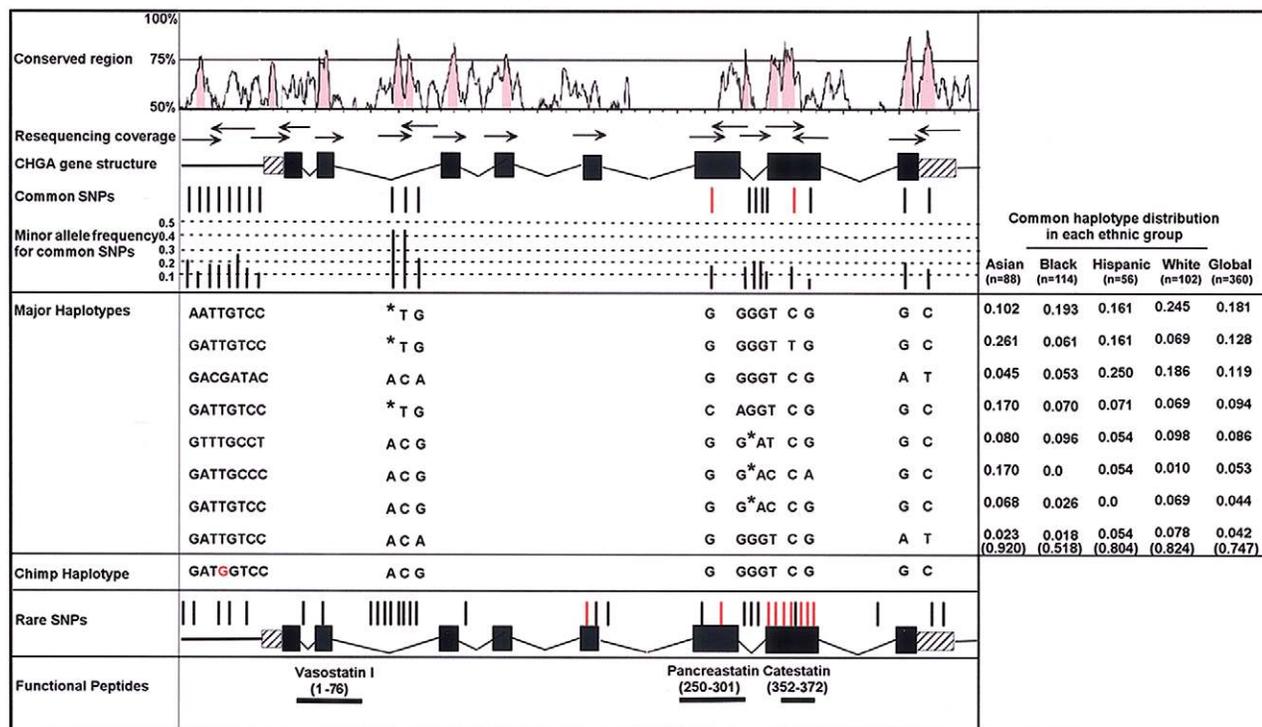
Figure 1 Resequencing strategy and identified variants. Sequences conserved between mouse and human *CHGA* were visualized with VISTA (Mayor et al. 2000). Location of common (*upper*) and rare (*lower*) SNPs relative to exons and conserved noncoding sequences is indicated by position. Red rods represent nonsynonymous SNPs, and black rods represent synonymous SNPs. Nucleotides in red in the chimpanzee haplotype indicate the minor allele in the human sequence. Computationally reconstructed haplotypes are indicated, along with their relative frequencies in ethnogeographic groups within our sample population. Nucleotide deletions in haplotype sequences are indicated by an asterisk (*).

influence *CHGA* gene function in our clinically focused samples. Consistent with African origins for modern humans, the eight major haplotypes account for only 51.8% of all haplotypes in African Americans but account for 92% in the Asian sample. (The F_{ST} for the *CHGA* proximal promoter among four populations is 0.035, which is significantly different from 0 [$P < .0001$]. This is attributable to the difference between the Asian sample and the other three populations: pairwise differences between populations for Asian versus black, Hispanic, and white are independently significant: $F_{ST} = 0.062$, 0.125, and 0.057, respectively [$P < .0001$], but differences among black, Hispanic, and white populations are not significant.)

The unusually high nucleotide diversity in the proximal promoter allowed us to examine this interval in greater detail. PHASE inferred eight haplotypes in the *CHGA* promoter region (table 2), six of which appeared to be relatively common (>4%). Four promoter SNPs in tight LD with each other (−1014, −988, −462, and −89; see table B [online only]) were common in the general population (although they were found in <5% of Asians), including one SNP (−988) for which the ancestral allele (in common with chimpanzee, bonobo,

and gorilla) is the minor allele in all populations. Three of these SNPs (−1014, −988, and −462) are in absolute LD with each other ($D' = 1$; $\Delta^2 = 1$), whereas the fourth (−89) shows complete but not absolute LD with the other three SNPs ($D' = 1$; $\Delta^2 = 0.744$). To illustrate relationships among the promoter haplotypes, we constructed haplotype networks through use of Arlequin (fig. 2A). The haplotype MST structure showed that the four linked SNPs divided *CHGA* promoter haplotypes into two clusters. The haplotype clusters that differ at these linked sites define a deep division in the human lineage. It is interesting that the chimp promoter haplotype was located between the two clusters, one step closer to the cluster that did not contain minor alleles of the four SNPs. It is surprising that haplotypes that retain the ancestral SNP allele at −988 occur at only a 20% frequency in the current general population. The high π value and divergent haplotype structure in the *CHGA* promoter could suggest effects of balancing selection, as was shown for the *cis*-regulatory region of *CCR5* ($\pi = 0.0021$) (Bamshad et al. 2002).

To determine whether the population structure of *CHGA* promoter variants is significantly different from expectation under selective neutrality, we applied sev-

Table 1

Nucleotide Diversity (π) in Each Region of *CHGA* among Four Populations

SEQUENCED REGION (NO. OF BASES SEQUENCED)	π IN POPULATION (TOTAL NO. OF SEGREGATING SITES)[a]				
	Asian (*n* = 88)	Black (*n* = 114)	Hispanic (*n* = 56)	White (*n* = 102)	Global (*n* = 360)
Coding (1,371)	.0009 (8)	.0009 (10)	.0009 (8)	.0007 (7)	.0009 (17)
Promoter (1,175)	.0012 (8)	.0022 (11)	.0023 (10)	.0023 (9)	.0021 (13)
5′UTR (213)	.0000 (0)	.0000 (0)	.0000 (0)	.0000 (0)	.0000 (0)
Intron (2,556)	.0011 (9)	.0012 (19)	.0009 (11)	.0011 (12)	.0011 (22)
3′UTR (410)	.0003 (1)	.0015 (3)	.0011 (1)	.001 (1)	.0008 (3)
Total (5,725)	.0007 (26)	.0009 (43)	.0008 (30)	.0008 (29)	.0008 (55)

[a] π was calculated as described by Tajima (1989).

eral well-described tests, including Tajima's *D* (Tajima 1989); Fu's *Fs,* Fu and Li's *D** and *F**, Fu and Li's *D* and *F,* and Fay and Wu's *H* test (Fu 1996; Fay and Wu 2000); and the HKA test (Hudson et al. 1987). The resulting statistics do not reach the .05 significance level for derived *P* values but are close in several cases (table 3), including a positive Tajima's *D* and Fu's *Fs* in white subjects. This is consistent with—but does not prove—selection acting on the promoter variants we identified. In protein coding sequence, we observed 11 replacement polymorphisms in the four populations but no fixed replacements between human and chimpanzee. This is significant by the McDonald-Kreitman test (McDonald and Kreitman 1991) with $P < .001$ (the test is independently significant at the .01 level for each ethnic group).

To test the functional significance of common variations in *CHGA,* we first examined the influence of individual common polymorphisms on the expression of *CHGA* gene products in vivo (fig. 2*B*). We looked at associations of SNPs across *CHGA* with four plasma CHGA peptide fragments in 102 subjects for whom we had both physiologic and gene resequencing data. One-way ANOVA with Bonferroni correction for multiple comparisons identified significant association with plasma CHGA for only the three SNPs (−1014, −988, and −462) that presented absolute LD with each other in the promoter region, suggesting a haplotype-specific

effect on plasma levels. Minor-allele homozygotes had significantly elevated levels of the two most abundant plasma CHGA peptides, compared with heterozygotes or major-allele homozygotes.

To test further the functional significance of common variation in the promoter region, we assayed expression of *CHGA* promoter haplotype-specific reporter constructs (fig. 2*C*). We placed the eight inferred promoter haplotypes upstream of a luciferase reporter and assayed expression in PC12 cells, which model a normal site of *CHGA* expression. It is interesting that the two common haplotypes containing minor alleles at the four complete-LD sites showed significantly lower expression than all the others, including chimpanzee. Haplotypes 3 (GACGATAC) and 6 (GACGATCC) are also significantly different from each other in expression level but differ in sequence only at −89. These results indicate that SNP −89 and at least one of the three SNPs in absolute LD are functionally significant.

To determine which of the three SNPs in absolute LD affect promoter activity, we mutated each of these sites in the promoter-reporter constructs for high-expressing haplotype 1 and low-expressing haplotype 6 (fig. 2*D*). For replacements on haplotype 1, only mutation of −462 (1−462A) shows significant decrease of promoter activity. For replacements on low-expressing haplotype 6, mutation of either −462 (6−462G) or −988 (6−988T) sig-

Table 2

Haplotype Distribution in the *CHGA* Promoter Region among Four Populations

| PROMOTER HAPLOTYPE NUMBER	NUCLEOTIDE AT POSITION								FREQUENCY (NO. OF CHROMOSOMES) IN POPULATION				
	−1106	−1018	−1014	−988	−462	−415	−89	−57	Asian (*n* = 88)	Black (*n* = 114)	Hispanic (*n* = 56)	White (*n* = 102)	Total (*n* = 360)
1	G	A	T	T	G	T	C	C	.466 (41)	.211 (24)	.321 (18)	.265 (27)	.306 (110)
2	A	A	T	T	G	T	C	C	.102 (9)	.272 (31)	.196 (11)	.255 (26)	.214 (77)
3	G	A	C	G	A	T	A	C	.045 (4)	.123 (14)	.268 (15)	.206 (21)	.15 (54)
4	G	A	T	T	G	C	C	C	.261 (23)	.158 (18)	.071 (4)	.078 (8)	.147 (53)
5	G	T	T	T	G	C	C	T	.102 (9)	.105 (12)	.107 (6)	.176 (18)	.125 (45)
6	G	A	C	G	A	T	C	C	0 (0)	.114 (13)	.036 (2)	0 (0)	.042 (15)
7	G	A	T	T	G	C	C	T	0 (0)	.018 (2)	0 (0)	.02 (2)	.011 (4)
8	G	T	T	T	G	C	C	C	.023 (2)	0 (0)	0 (0)	0 (0)	.006 (2)
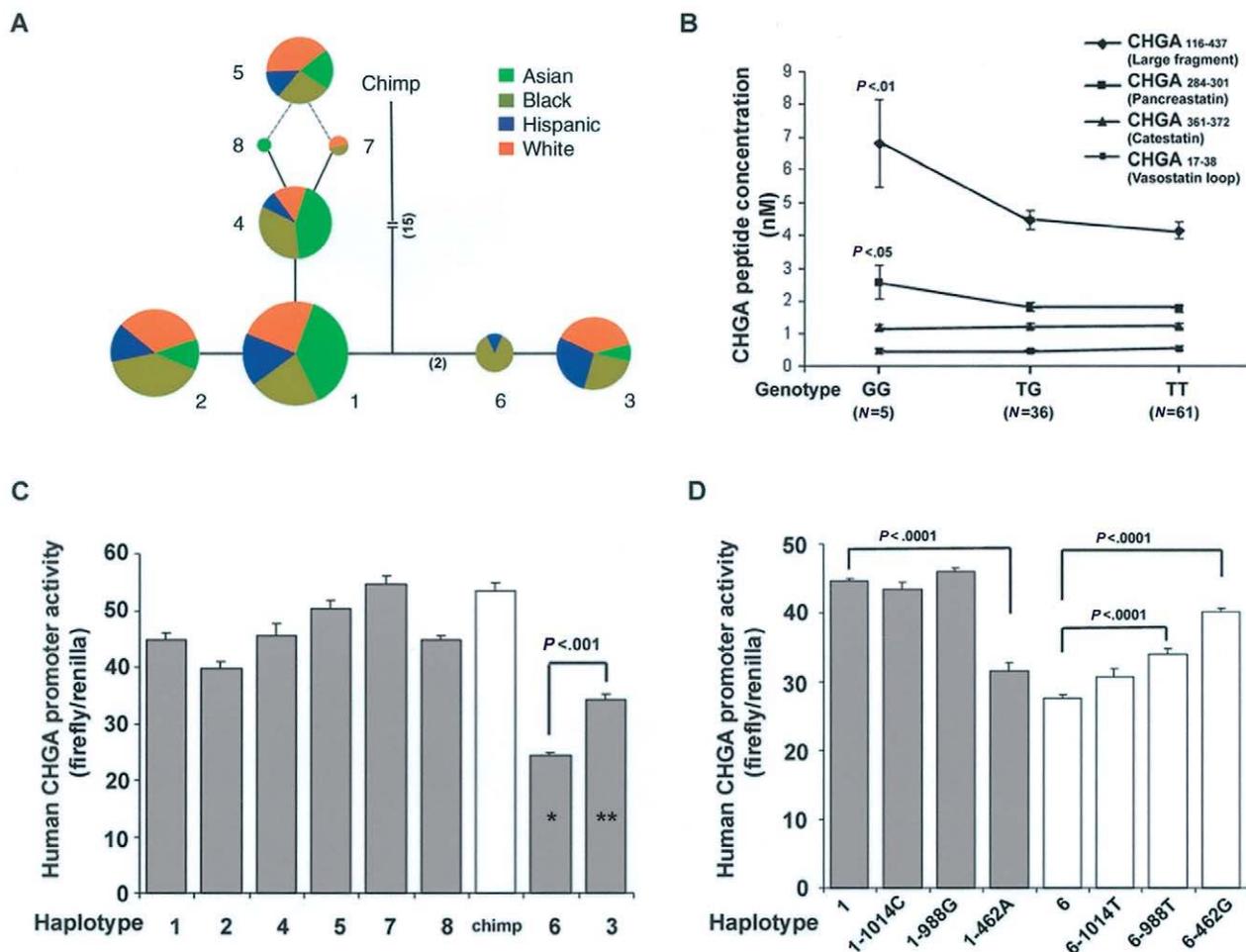
Figure 2   Functional variation in the *CHGA* promoter. *A,* Promoter haplotypes, each represented by a circle whose area represents the overall frequency of that haplotype in the sample. Each haplotype number corresponds to haplotype numbers in table 2. Each circle is subdivided to show the proportion of the individual haplotype frequency found in each of the four populations as represented by the indicated colors. Dashed lines indicate alternative topologies of equal length. Lines connecting haplotypes represent one nucleotide substitution, except where noted in parentheses. *B,* Association of *CHGA* proximal promoter SNP genotype (G-988-T) with in vivo plasma CHGA peptide levels in 102 subjects. All CHGA peptides levels are expressed as mean ± SEM. Significant differences could be observed in two peptide fragments (large fragment, $CHGA_{116-457}$; and pancreastatin, $CHGA_{284-301}$) between minor-allele homozygotes and the other two groups. N = number of subjects for each genotype group. The allele frequencies were 22.5% for G and 77.5% for T. The genotypes were in Hardy-Weinberg equilibrium ($\chi^2 = 0.011$; $P = .91$). *C,* In vitro haplotype-specific *CHGA* promoter activity assay. Two haplotypes (3 and 6) showed a marked decrease in promoter activity compared with the other four common promoter haplotypes, two rare haplotypes, and chimp haplotype. Haplotype numbers correspond to haplotype numbers in table 2. There are also significant differences in promoter activity between haplotypes 3 and 6. *$P < .0001$ between haplotype 6 and other haplotypes. **$P < .001$ between haplotype 3 and the other haplotypes except haplotype 6. *D,* In vitro mutated haplotype activity assay in the *CHGA* promoter. Each mutated promoter haplotype was derived from either haplotype 1 or haplotype 6 (see table 2).

nificantly increases expression, with −462G conferring the largest increase.

To test the functional significance of the rare amino acid replacements we found in the catestatin peptide sequence, we synthesized wild-type and variant peptides and assayed their potency for inhibition of nicotinic cholinergic-stimulated catecholamine release from chromaffin (PC12) cells (fig. 3). The Gly364Ser substitution alters a site that is otherwise absolutely conserved among seven

mammalian species for which sequence is available but occurs in 11 of 180 subjects. This substitution in synthetic catestatin results in a 4.7-fold loss of potency in our assay. Pro370Leu occurred in 2 of 180 subjects; it is interesting that 370Leu is the normal allele in all reported nonprimate mammals. This substitution results in a 2.3-fold gain of potency of the synthetic peptide (fig. 3A and 3B). It is interesting that the Gly364Ser variant is distributed across other ethnic groups (five are Asian samples, five

Table 3

Neutral Selection Tests in the *CHGA* Promoter Region among Four Populations

Population	Tajima's *D*	Fu's *Fs*	Fu and Li's *D**	Fu and Li's *F**	Fu and Li's *D*	Fu and Li's *F*	Fay and Wu's *H*
Asian (*n* = 88)	−.252 (.44)	.644 (.19)	1.268 (.09)	.891 (.19)	1.292 (.08)	.906 (.16)	−4.486 (.016)
Black (*n* = 114)	.647 (.22)	.472 (.148)	.061 (.36)	.321 (.35)	.050 (.36)	.318 (.38)	−1.322 (.14)
Hispanic (*n* = 56)	.766 (.16)	.953 (.159)	.136 (.39)	.408 (.38)	.118 (.34)	.403 (.36)	−.529 (.23)
White (*n* = 102)	1.460 (.074)	2.757 (.068)	.564 (.45)	1.037 (.14)	.568 (.16)	1.049 (.13)	−1.594 (.1)
Global (*n* = 360)	.498 (.24)	.059 (.125)	−.703 (.15)	−.293 (.38)	−.715 (.15)	−.300 (.40)	−1.867 (.09)

NOTE.—Nominal *P* values are given in parentheses for each test.

are white samples, and one is a Hispanic sample) but is absent from the African American sample ($P < .01$; χ^2 test), whereas the Pro370Leu variant occurred only in African American samples.

To determine how these variants could influence peptide potency, we modeled the impact of amino acid substitutions on the NMR structure (PDB 1lv4) of human catestatin that we had recently determined. Homology modeling suggests that Gly364Ser distorts the peptide backbone in two locations, by 0.36 Å in the middle loop near the site of substitution and by 1.48 Å in the carboxy-terminal strand, which is adjacent in the three-dimensional structure (fig. 3*C, left*). By contrast, the Pro370Leu substitution model predicts a 0.59-Å shift in the middle loop but no shift in the carboxy-terminal strand near the site of substitution (fig. 3*C, right*).

Discussion

CHGA plays crucial roles in the sympathoadrenal system, both in the formation of catecholamine secretory vesicles and in the regulation of catecholamine release by nicotinic cholinergic stimuli (Taupenot et al. 2003). Here, we find both quantitative variation in expression level mediated by common promoter haplotypes and qualitative variation in rare catestatin peptide polymorphisms that alter its inhibitory potency several fold. We find 13 promoter SNPs that resolve into six relatively common haplotypes (frequencies >4%). These 5′ proximal haplotypes differ quantitatively in the ability to promote transcription, and one of the functionally variant SNP alleles (G-988-T) in the low-expression haplotype is an ancestral allele that has diminished in allele frequency in a region of likely natural selection. The functional variation we observed could be a substrate for environmental selective pressures dependent on catecholaminergic function.

Tests of selective neutrality provide modest support for recent selection on *CHGA* promoter haplotypes in white and Asian subjects. Tajima's *D* and Fu's *Fs* tests show positive statistics just above traditional significance thresholds in white subjects (with *P* values of .074 and .068, respectively), whereas Fu and Li's *D* and *D** tests are nearly significant and Fay and Wu's *H* test is significant in Asian subjects (with *P* values of .08, .09, and .016, respectively). The difference between populations probably reflects the near absence of haplotypes 3 and 6 in Asian samples, increasing the proportion of chromosomes containing the nonancestral allele at position −988. We do not see significant deviation from neutrality in the other populations, but the statistical methods are relatively conservative and have power to detect selection only under limited circumstances.

Our results suggest that selection in modern humans favors haplotypes that impart a moderate range of *CHGA* expression. It is interesting to note that the most extreme promoter haplotypes for functional expression (6 and 7) are infrequent and are intermediates that link more-moderate and more-common promoter haplotypes in the minimum spanning network. In the coding sequence, the McDonald-Kreitman test showed significant departure from neutrality, because of the lack of fixed replacements. Consistent with purifying selection against replacements, we find that two relatively infrequent replacement polymorphisms in the catestatin region alter peptide potency several fold. Taken together with our functional studies, our molecular evolutionary data suggest the importance of maintaining *CHGA* expression within an approximately twofold range, but more-sensitive and more-powerful approaches will likely be needed to confirm the influence of selection in shaping modern haplotypes.

In vitro promoter studies indicate that three of the promoter SNPs we identified have functional consequences. The −89 SNP is the only difference between promoter haplotypes 3 and 6, accounting for ~30% change in expression level. The −462 SNP accounts for another 30%–40% change, including most of the difference between haplotypes 1 and 6. It is interesting that the SNP at −988 had no effect on haplotype 1 but accounted for a slight increase in expression from haplotype 6. This may suggest an epistatic interaction among SNPs within the variant promoter haplotypes. The −462 and −89 polymorphisms each sit within consensus sites for known transcription factor families, identified computationally by rVista (Loots et al. 2002) or TESS (Schug and Overton 1997). These include TCF1α and GATA sites at −462 and one of several potential AP-2 sites at
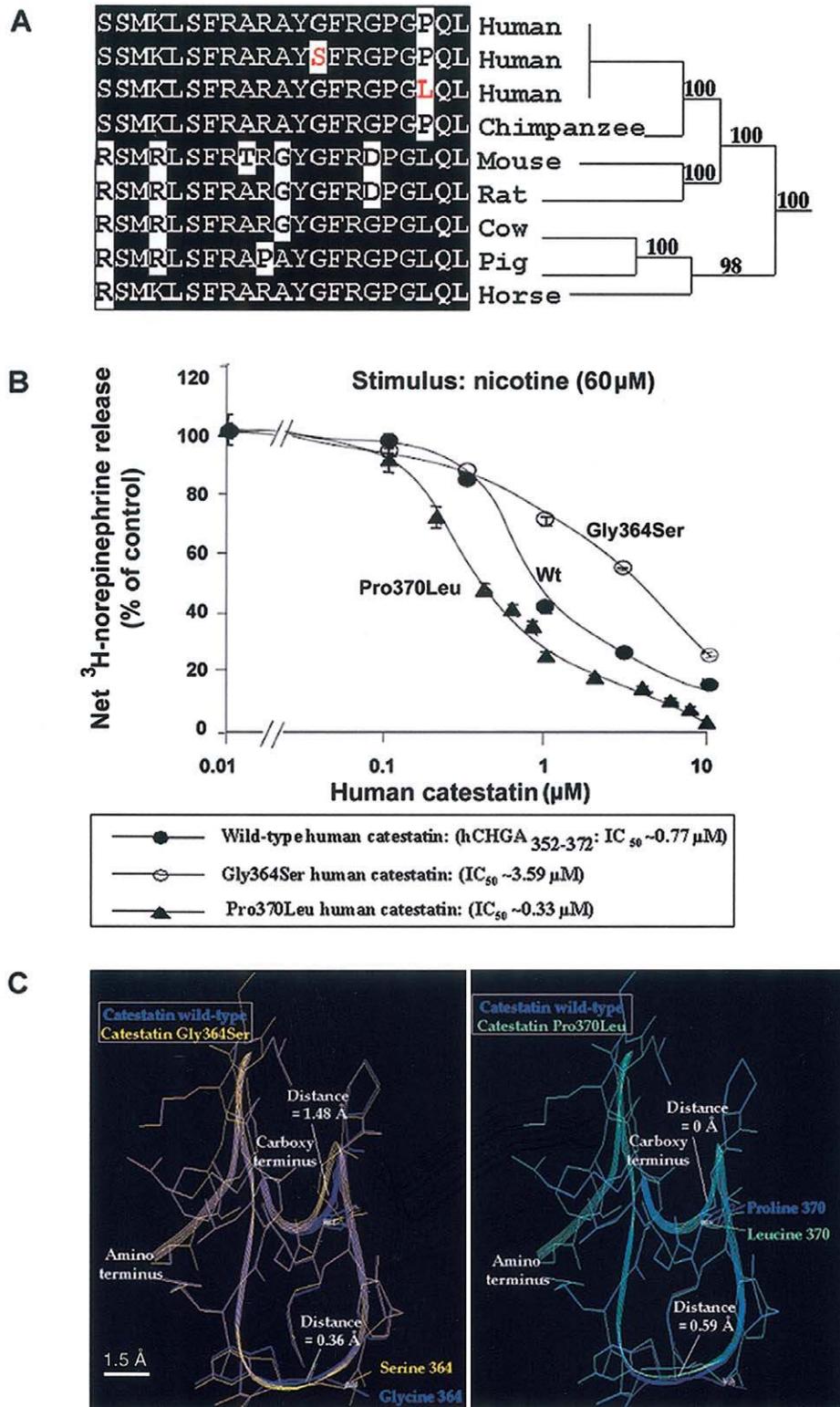
Figure 3   Catestatin peptide variants altering cholinergic inhibition and predicted structure. *A*, Peptide sequence alignment from several species (and the corresponding catestatin region), showing the extent of sequence conservation at Gly364Ser and Pro370Leu (*red, second and third rows*). Note that 370Leu is found in all nonprimate species available. Phylogenetic relationships and bootstrap values are taken from a published multiple gene comparison (Murphy et al. 2001). *B*, Altered efficacy of nicotinic inhibition by variant peptides in dose response from 0.1 to 10 μM, showing functional significance to each change, but in opposite directions. *C*, Homology modeling, predicting altered three-dimensional structure of Gly364Ser (*left*) and Pro370Leu (*right*) variants. Point mutants were aligned to the wild-type backbone template and then subjected to energy-minimization/ homology-modeling using SWISS-MODEL at the ExPASy Web site and Swiss-PDBviewer ("DeepView") for visualization and manipulation (Peitsch 1995).
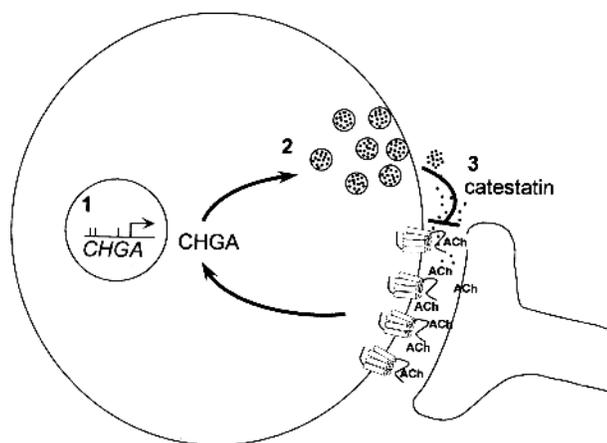
Figure 4   1-2-3 model for influence of *CHGA* polymorphisms on catecholamine storage and release. *1,* Common promoter haplotypes affect the transcriptional level of the *CHGA* gene in chromaffin cells. *2,* CHGA protein levels quantitatively affect pool size of catecholamine-chromogranin vesicles. *3,* Variant catestatin peptides alter the release-dependent feedback inhibition of nicotinic-stimulated release. This feedback loop could explain the divergent effects of promoter polymorphisms on chromaffin cell *CHGA* expression and plasma levels.

−89. However, occupancy and differentiation of these sites need to be demonstrated in vivo before a precise transcriptional mechanism for the altered expression phenotypes can be confirmed.

Two relatively infrequent amino acid replacement cSNPs in the catestatin peptide ($CHGA_{352-372}$) produce quite different effects on catestatin potency, ultimately differing by 10.8-fold in their potency to inhibit nicotinic stimulation of catecholamine release from chromaffin cells. The Gly364Ser variant occurs at a highly conserved site among mammalian catestatins. The 364Ser variant we discovered reduces catestatin activity ~4.7-fold. In contrast, the 370Leu variant is the normal allele in all mammalian species sequenced to date, except human and chimpanzee; thus, the Pro370Leu allele that increases catestatin activity ~2.3-fold is a reversion to the usual amino acid seen among mammals.

Our data demonstrate that both common and rare variants contribute to functional polymorphism at *CHGA* and suggest a model (fig. 4) for feedback regulation of catecholamine and chromogranin release mediated by catestatin. Correlations between quantitative functional alterations and allele frequencies suggest that the level of CHGA and catestatin is under selection but that different activities of CHGA may be under different selective pressures. Although larger cohort sizes will need to be examined to determine the impact of these alleles on complex phenotypes such as blood pressure, our results clearly reinforce a likely role for *CHGA* variation in intermediate phenotypes governing catechol-amine physiology and suggest that current haplotype diversity at *CHGA* has been shaped by selective pressure in the modern human lineage.

Electronic-Database Information

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www
.ncbi.nlm.nih.gov/Omim/ (for CHGA)
Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/
primer3_www.cgi (for design of PCR primers)
Protein Data Bank, http://www.rcsb.org/pdb/ (for the NMR
structure of catestatin, entry "1lv4")
SWISS-MODEL at ExPASy, http://www.expasy.org/swissmod/
SWISS-MODEL.html
UCSC Genome Bioinformatics, http://genome.ucsc.edu/

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