

# Differential Enzymatic Activity of Common Haplotypic Versions of the Human Acidic Mammalian Chitinase Protein\*<sup>§</sup>

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Mouse models have shown the importance of acidic mammalian chitinase activity in settings of chitin exposure and allergic inflammation. However, little is known regarding genetic regulation of AMCase enzymatic activity in human allergic diseases. Resequencing the *AMCase* gene exons we identified 8 non-synonymous single nucleotide polymorphisms including three novel variants (A290G, G296A, G339T) near the gene area coding for the enzyme active site, all in linkage disequilibrium. AMCase protein isoforms, encoded by two gene-wide haplotypes, and differentiated by these three single nucleotide polymorphisms, were recombinantly expressed and purified. Biochemical analysis revealed the isoform encoded by the variant haplotype displayed a distinct pH profile exhibiting greater retention of chitinase activity at acidic and basic pH values. Determination of absolute kinetic activity found the variant isoform encoded by the variant haplotype was 4-, 2.5-, and 10-fold more active than the wild type AMCase isoform at pH 2.2, 4.6, and 7.0, respectively. Modeling of the AMCase isoforms revealed positional changes in amino acids critical for both pH specificity and substrate binding. Genetic association analyses of *AMCase* haplotypes for asthma revealed significant protective associations between the variant haplotype in several asthma cohorts. The structural, kinetic, and genetic data regarding the AMCase isoforms are consistent with the Th2-priming effects of environmental chitin and a role for AMCase in negatively regulating this stimulus.

Chitin, a linear polymer of the sugar *N*-acetylglucosamine, is ubiquitous in nature and comprises a structural component in the cell wall of fungi, the exoskeleton of arthropods such as insects and crustaceans, and in the eggs and pharyngeal organs of parasitic nematodes (1–5). Chitinases are evolutionarily ancient enzymes that hydrolytically cleave the chitin polymer into di- and trisaccharides. Chitinases serve functionally diverse roles across species, including nutrient scavenging, structural remodeling of chitin constituents, parasitism, and innate immunity (6, 7).

Despite the absence of endogenous chitin, humans express two active chitinases, designated acidic mammalian chitinase (AMCase)<sup>2</sup> and chitotriosidase (6). Chitotriosidase (CHIT1) is expressed by phagocytic cells in humans and extremely high levels are seen in lysosomal storage diseases, such as Gaucher disease (8). The high frequency of a null duplication polymorphism in the *CHIT1* gene triggered the search for other lung chitinases that may compensate for the loss of CHIT1 enzymatic activity. This search resulted in the discovery of the AMCase enzyme in both mice and humans. The AMCase enzyme has been suggested to be involved in asthma due to the finding that its expression was strongly up-regulated in response to sensitization and challenge in an ovalbumin mouse model of asthma. The expression of AMCase was found to be induced by interleukin-13, a cytokine implicated in many of the effects of human asthma (9, 10). Moreover, the chitinase activity of AMCase was found to be causative in allergic inflammation, as inhibition of AMCase activity greatly reduced Th2-dependent airway inflammation and bronchial hyperresponsiveness in these mice (10). Intriguingly, this report also found

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<sup>2</sup> The abbreviations used are: AMCase, acidic mammalian chitinase; SNP, single nucleotide polymorphism; CHIT1, chitotriosidase; SAGE, Study of African Americans, Asthma, Genes, and Environments; GALA, Genetics of Asthma in Latino Americans; CHIRAH, Chicago Initiative to Raise Asthma Equity; ACRN, Asthma Clinical Research Network; CHS, Children's Health Study; 4-MU-chitobioside, 4-methylumbelliferyl- $\beta$ -*N,N'*-diacetylchitobioside.

AMCase to be induced strongly in both lung epithelial cells and macrophages among fatal asthmatics (10). However, a more recent report implicated chitin itself as a pattern recognition molecule stimulating the tissue accumulation of innate immune cells associated with asthma, such as eosinophils and basophils (11). Importantly, this study found pretreatment of the polymeric chitin with recombinant AMCase attenuated the inflammatory effects of chitin (11). The results were confirmed *in vivo*, showing a strong reduction in the inflammatory effects of chitin in mice overexpressing AMCase in the airway epithelium (11).

Despite the observed importance of chitinase activity in animal models of asthma, little is known regarding regulation of chitinase activity in human disease. The only genetic studies of the *AMCase* gene, conducted in Indian and German populations, have found the gene to be polymorphic and observed significant associations between several polymorphisms and asthma disease status (12, 13). In fact, the Indian study found the associated promoter polymorphisms significantly affected transcription levels *in vitro* (13). The variants associated with asthma in the German study result in amino acid changes, raising the possibility that coding *AMCase* polymorphisms may influence chitinase activity and potentially disease risk (12). Biochemical analysis of the human AMCase protein has revealed the enzyme does cleave chitin substrates and that its activity is modified by pH and salt concentration (7). In this report we have explored the role that genetic variation plays in regulation of AMCase enzymatic activity. Herein we describe all common coding variations and haplotypes in the human *AMCase* gene. We have biochemically characterized two haplotypic isoforms of the AMCase enzyme differing in amino acids close to the enzyme active site and assessed their effect on the three-dimensional structure of the enzyme. Last, we explored whether the gain-of-function haplotype discovered was associated with asthma risk in several United States ethnic groups.

## EXPERIMENTAL PROCEDURES

### *AMCase* Sequencing, Linkage Disequilibrium, and Haplotype Analysis

All *AMCase* exons (12) and exon-intron boundaries (20 bp from either end of the exon) were sequenced in a SNP Discovery panel, which contains 72 subjects with asthma who self-identified as African American, Puerto Rican, or Mexican. Variants identified, sequencing details, and primers are listed in the supplemental data (Tables S1 and S2). Linkage disequilibrium patterns were determined by using the  $r^2$  statistic, using the program haploview (Table S3) (14). Haplotypes and their frequencies were determined with the program HAPSTAT (15).

### *AMCase* Expression Plasmid Cloning

The full-length *AMCase* cDNA was fused to an Fc purification tag and cloned into the pMIB-V5-His-(A) vector (Invitrogen). The Fc purification tag did not contain the hinge region to prevent dimerization of the proteins. Successive rounds of *in vitro* mutagenesis were conducted on the *AMCase* expression construct using the Stratagene QuikChange kit, to generate both the variant and wild type *AMCase* haplotypes. Detailed

methods are listed in the supplemental data. PCR cloning and mutagenesis primers for all of these cloning and mutagenesis steps are contained in Tables S4 and S5.

### Expression and Purification

In parallel, High Five insect cells (Invitrogen) were transfected with the pMIB-V5-His-(A)-*AMCase*-Fc vectors containing both the variant (GATGAGCG) and wild type (AGGGAATT) haplotypes, and mock transfection. Blastidicin was used to select for stable cell lines over a 3-week period. These cell lines were grown in culture dishes for 5 days and AMCase was purified from the supernatants using Immobilized Protein G resin (Pierce Biotechnology). A detailed purification protocol is described under the supplemental data. Eluted fractions were run on SDS-PAGE gels to ensure the purity of the AMCase protein. AMCase protein concentration was determined with the Micro BCA assay (Pierce Biotechnology) according to the manufacturer's instructions.

### *AMCase* Enzymatic Assays

**General**—AMCase enzymatic activity was determined using the synthetic chitin substrate 4-MU-(4-deoxy)chitobiose (16). All enzymatic reactions for both determination of optimum pH and kinetic parameters were conducted in a volume of 100  $\mu$ l, containing 100 ng of AMCase protein in McIlvaine's buffer. All reactions were conducted in triplicate in 96-well fluorescent spectroscopy plates (Sigma) with 4-methylumbelliferone also loaded as a standard. Reactions for both pH and kinetic assays were incubated in a SpectraMax Gemini XS fluorescence plate reader for 20 min at 37 °C. Reactions were stopped by the addition of 120  $\mu$ l of 1 M glycine/NaOH, pH 10.5, to reaction wells. Plates were then immediately read on the SpectraMax fluorometer at an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

***AMCase* pH Assays**—We tested the enzymatic activity of both AMCase isoforms in McIlvaine buffer at pH values of 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 4.6, 5.0, 5.4, 6.0, 7.0, and 8.0 to determine the AMCase pH profiles. Enzymatic activity at all pH values were tested at a substrate concentration of 75  $\mu$ M.

***AMCase* Kinetic Reactions**—Michaelis-Menten kinetic curves were generated by performing AMCase enzymatic reactions with the substrate 4-MU-(4-deoxy)chitobiose at concentrations of 10, 25, 50, 75, 100, 150, and 200  $\mu$ M. Kinetic assays for both isoforms were performed at pH values of 2.2, 4.6, and 7.0. Reactions were conducted as described under "General."

***AMCase* Enzymatic Data Analysis**—Enzymatic activity in pH profiles was displayed as a percent of activity at the pH where the isoform was most active (pH 4.6 for both isoforms). The pH profiles displayed in Fig. 3A are the result of a single experiment representative of at least two independent experiments.

For determination of enzyme velocities the mean fluorescence units detected from the triplicate assays were converted to picomoles of product produced by extrapolation using the 4-MU standard curves generated on each plate, and divided by reaction time and enzyme amount. Enzyme velocities divided by the amount of enzyme in the reaction were plotted *versus* the substrate concentration and fitted to the Michaelis-Menten

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equation by non-linear regression to determine the kinetic parameters,  $K_m$  and  $K_{cat}$  of each experiment, using the program Graphpad Prism 4.0. The kinetic parameters listed herein are the mean of four independent experiments for the analysis at pH 4.6 and two independent experiments for the analysis at pH 2.2 and 7.0. Statistical comparison of the kinetic parameters was by two-sample two-tailed  $t$  test assuming equal variances.

### Modeling of the Wild Type and Variant AMCase Isoforms

The recombinant protein used to generate the AMCase structure contains the variant haplotype amino acid changes (17). To investigate the influence of these changes on the overall structure we generated initial models of AMCase using the native AMCase crystal structure Protein Data Bank code 3FXV (A chain resolution, 2.0 Å) and the SWISSPROT server (18). To generate the wild type model the aspartate at position 45 was converted to an asparagine, asparagine at position 47 was converted to an aspartate, and the methionine at position 61 was converted to an arginine. Both the native and modified model were subjected to energy minimization using the macromolecular modeling package CHARMMing tool in the default settings (19). The structural implications were analyzed using MacPyMOL.

### Genetic Study Populations and Genotyping Analysis

SNPs G339T, G461A, A531G, G1172A, T1218G, and G1452T were genotyped in the Study of African Americans, Asthma, Genes, and Environments (SAGE) and Genetics of Asthma in Latino Americans (GALA) study subjects. The SAGE study includes 264 asthmatic cases and 176 matched controls, recruited in the San Francisco Bay Area. The Genetics of Asthma in Latino Americans (GALA) study recruited 301 Mexican and 399 Puerto Rican asthmatics (probands) and their biologic parents from 4 different clinical sites: Mexico City/San Francisco (Mexican) and Puerto Rico/New York (Puerto Ricans). African American asthmatic cases from the Chicago Initiative to Raise Asthma Equity (CHIRAH) and the Asthma Clinical Research Network (ACRN) cohorts were genotyped for the G339T AMCase SNP. In addition, we genotyped the G339T AMCase SNP in 259 African American (asthmatic cases = 61, controls = 198) and 456 Latino (asthmatic cases = 138, Controls = 318) subjects from the Children's Health Study (CHS). All genotyping methods and primers are described under supplemental data (Table S6). All study groups are described in detail in the online supplement with characteristics listed in Tables S7 and S8. Local Institutional Review Boards approved all the studies, and all subjects provided written, age-appropriate informed consent or assent.

### Genetic Statistical Analyses

The  $\chi^2$  goodness-of-fit test was used to determine whether the individual AMCase SNPs were under Hardy Weinberg equilibrium. All SNPs genotyped conformed to Hardy Weinberg equilibrium ( $p > 0.01$ ) in all five study groups tested. Standard logistic regression was used to test association between AMCase SNP alleles and asthma disease status in the SAGE, CHIRAH, and ACRN cohorts. Age and gender were both included as covariates in the logistic regression models.

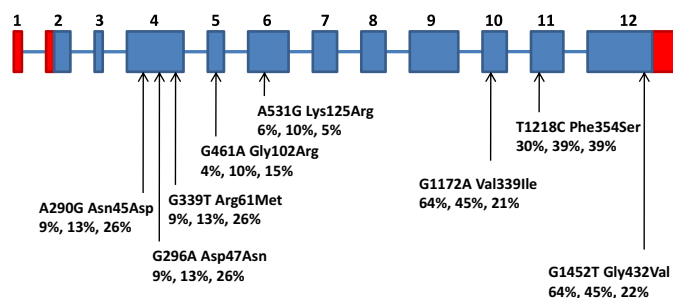


FIGURE 1. The structure of the human AMCase gene, with accompanying common non-synonymous SNPs present among Mexicans, Puerto Ricans, and African Americans. Exons are identified by blue and red block structures and numbered accordingly. The red portion of exons represent untranslated regions and the blue represent the coding portion of exons. All variant mRNA and amino acid positions are reported with regard to mRNA sequence uc001eas.1 (UCSC Genome Browser). Percentages listed refer to minor allele frequencies in Mexicans, Puerto Ricans, and African Americans, respectively. The minor allele and amino acid is listed second.

Additionally, individual ancestry estimates (see supplemental data) were covariates in the SAGE and CHIRAH analyses. All statistical analyses in the three populations were performed using STATA 8.0 S/E statistical software (College Station, TX). FBAT was used to assess the association between individual SNPs with asthma in the GALA families (20). Replication analysis in the CHS subjects was performed by logistic regression assuming an additive genetic model. Development of the model and covariates included are described under the supplemental data. CHS analysis was performed in SAS version 9.1 (SAS Institute Inc., Cary, NC).

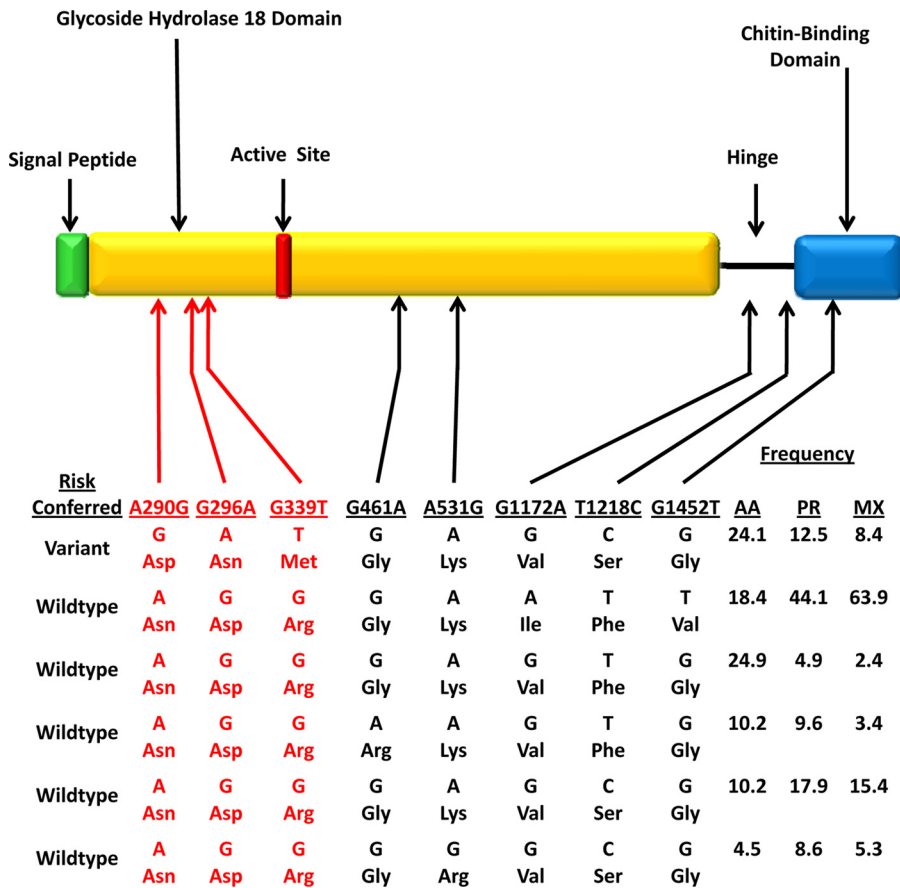
## RESULTS

**Identification of Common AMCase Coding Variants and Linkage Disequilibrium Patterns**—To identify common genetic variants in the AMCase gene that may influence chitinase activity we resequenced all 12 AMCase exons in three diverse populations: African Americans, Puerto Ricans, and Mexicans. We identified 21 variants including four 5'-untranslated region variants, nine non-synonymous SNPs, four synonymous SNPs, a 3'-untranslated region SNP, and three intronic SNPs, of which nine were novel (Table S1). Among the non-synonymous SNPs there were eight variants that were common (>5% allele frequency) to all three racial groups SNPs: A290G, N45D; G296A, D47N; G339T, R61M; G461A, G102R; A531G, K125R; G1172A, V339I; T1218C, F354S; and G1452T, G432V (Fig. 1). Minimal linkage disequilibrium was exhibited between these variants with the exception of three novel SNPs, A290G, G296A, and G339T (Table S2). These three SNPs were inherited as a linkage disequilibrium block (pairwise  $r^2 = 1.0$ ) in all populations.

**Gene-wide AMCase Haplotypes**—To help determine which amino acid changes may be critical to enzymatic activity we determined their location within the AMCase protein domains. As observed from Fig. 2, the AMCase protein is composed of an N-terminal signal peptide, followed by the glycosyl hydrolase 18 domain, containing a highly conserved active site, linked to the chitin binding domain by a hinge region. Variants localized both within the chitin-binding and glycosyl hydrolase domains. Most interestingly, the three linked amino acid changes, R61M, N45D, and D47N, clustered in the glycosyl hydrolase domain in



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**FIGURE 2. AMCase protein domain structure and common haplotypes with frequencies in African Americans, Puerto Ricans, and Mexicans.** Haplotypes are defined as variant or wild type depending on whether they contained the minor or major alleles of the three linked SNPs (shown in red) in close proximity to the active site (A290G, G296A, G339T), respectively. The haplotype alleles for SNPs A290G and G296A are inferred based on the linkage disequilibrium patterns defined in resequencing. Haplotypes frequencies reported are for SAGE controls and GALA parents.

very close proximity to the active site. To determine the naturally occurring gene-wide *AMCase* haplotypes we genotyped these three SNPs and the other 5 coding *AMCase* SNPs in 176 African Americans, 602 Mexicans, and 798 Puerto Rican subjects. We then used these genotype calls to infer the *AMCase* gene-wide haplotypes using haplotype software Hapstat. We observed 6 different common (>5% frequency) haplotypes in African Americans and Latinos (Fig. 2). Interestingly, the variant alleles of the three linked variants near the AMCase active site (A290G, G296A, and G339T) only occurred on one of the six haplotype backgrounds, GATGAGCG. We hypothesized that the naturally occurring AMCase protein isoform encoded for by the GATGAGCG haplotype would display differential chitinase activity in comparison to any one of the other *AMCase* haplotypes, which contain the wild type alleles of the three variants in close proximity to the active site. As a comparison to the GATGAGCG haplotype, we selected the most common of these wild type allele containing haplotypes, AGGGAATT (African American, 18.4%, Mexican, 44.1%, Puerto Ricans, 63.9%), for expression and enzymatic analyses.

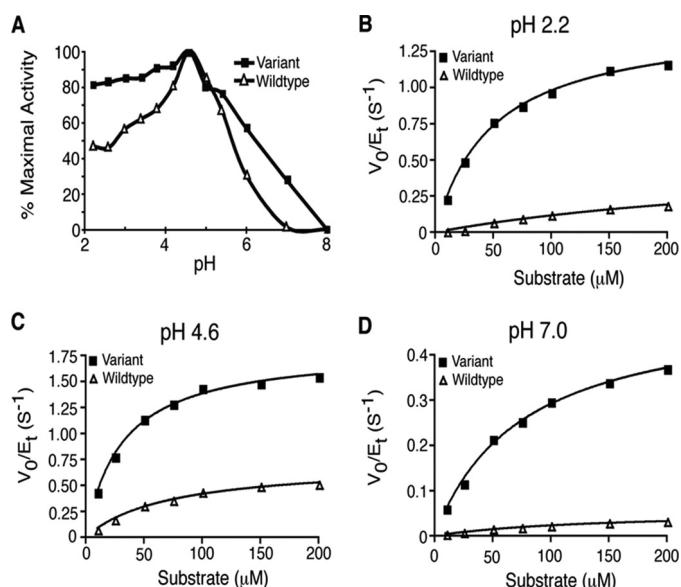
**Biochemical Analysis of Variant Human AMCase Isoforms—**Using an insect cell expression system and Fc affinity purification tag we expressed and purified both isoforms of the AMCase protein. Chitinase activity was assessed by enzy-

matic digestion of a synthetic chitin substrate. Although chitinase activity is commonly determined using fluorogenic substrates, such as 4-methylumbelliferyl- $\beta$ -*N,N'*-diacetylchitobioside (4-MU-chitobioside: two *N*-acetylglucoamine sugars linked to the fluorescent 4-MU molecule), AMCase enzymatic activity is inhibited at high substrate concentrations, thus precluding accurate determination of kinetic parameters with these substrates (16). Therefore, we used a chemically altered version of 4-MU-chitobioside, 4-MU-(4-deoxy)-chitobioside, which has been modified to prevent substrate inhibition, thus allowing accurate determination of enzyme kinetics (16). Previous work has shown the optimal pH of AMCase enzymatic activity is 4.6, with sharply declining activity at more acidic and basic pH values (7). We determined the pH profile of enzymatic activity for both isoforms of the protein over a range of pH values from 2.2 to 8.0, and corroborated that both isoforms display optimal enzymatic activity at pH 4.6 (7). We found that the variant isoform (GATGAGCG) retained near optimal activity at acidic pH values (Fig. 3A). In contrast, enzymatic

activity of the wild type isoform (AGGGAATT) declined sharply with decreasing pH, as previously reported for AMCase (Fig. 3A) (7). Furthermore, the variant isoform maintains 57 and 28% of optimal enzymatic activity at pH values of 6.0 and 7.0, respectively, whereas the wild type isoform has only 31% of optimal enzymatic activity at pH 6.0 and is essentially devoid of activity at pH 7.0.

We next kinetically characterized the AMCase isoforms to identify any differences in absolute enzyme activity between the variant and wild type AMCase isoforms. To conduct the kinetic analysis we selected three pH values, which reflect the three pH environments likely to contain AMCase in the human body: 1) pH 2.2, reflecting the stomach acidity where AMCase is highly expressed; 2) pH 4.6, reflecting the pH of lysosomes where some AMCase likely resides; and 3) pH 7.0, reflecting the pH of the airway lumen. AMCase isoforms were tested at a range of substrate concentrations from 10 to 200  $\mu$ M, and data tightly fit the Michaelis-Menten equation at all pH values for both isoforms ( $r^2 > 0.95$ ). The kinetic parameters determined for the two AMCase isoforms at pH 2.2 revealed a significant increase in enzymatic activity of the variant isoform versus the wild type isoform at both subsaturating (variant  $K_m = 48 \mu$ M, wild type  $K_m = 171.6 \mu$ M,  $p = 1.8 \times 10^{-3}$ ) and saturating substrate concentrations (variant  $K_{cat} = 1.43 \text{ s}^{-1}$ , wild type  $K_{cat} = 0.34 \text{ s}^{-1}$ ,

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**FIGURE 3. pH and kinetic characterization of AMCase isoforms.** AMCase activity was determined using the synthetic chitin substrate 4-MU-(4-deoxy)-chitobiose. *A*, pH activity profile of variant (GATGAGCG) and wild type (AGGGAATT) AMCase isoforms. Activity at each pH is expressed as a percentage of activity at optimum pH. Michaelis-Menten kinetic profiles of AMCase isoforms. *B*, assays conducted at pH 2.2. *C*, assays conducted at pH 4.6. *D*, assays conducted at pH 7.0. Substrate concentrations tested were 10, 25, 50, 75, 100, 150, and 200  $\mu\text{M}$ . Curves were fit to data by nonlinear regression using the Michaelis-Menten equation. All graph points are the mean of triplicate measurements and representative of multiple experiments.

**TABLE 1**

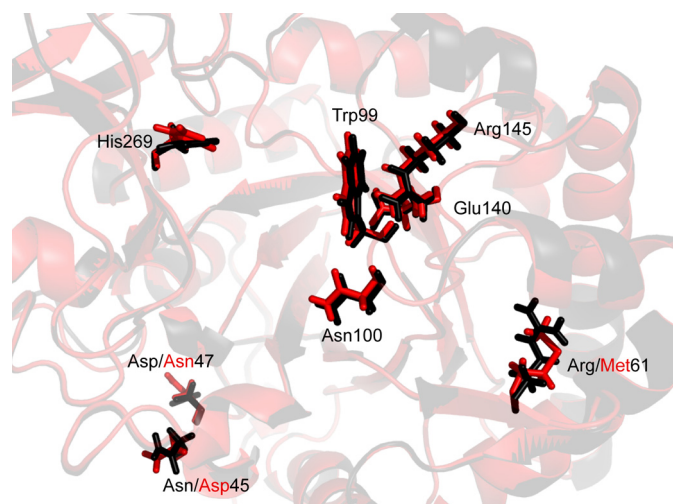
### Summary of the Michaelis-Menten kinetic parameters for AMCase isoforms at physiological pH values

Kinetic parameters are the best fit values from non-linear regression of data with the Michaelis-Menten equation. All data points are the mean of triplicates measurements, and kinetic parameters are the mean of at least two independent experiments. Numbers in parentheses are standard deviation of kinetic parameters. Statistical comparison of kinetic parameters conducted by Student's *t* test.

AMCase haplotypes	Kinetic parameters			
	$K_{\text{cat}}$	$p$ value	$K_m$	$p$ value
<b>pH 2.2</b>				
Variant GATGAGCG	1.43 (0.04)	$1.9 \times 10^{-3}$	48.01 (1.93)	$1.8 \times 10^{-3}$
Wild type AGGGAATT	0.34 (0.05)		171.6 (7.21)	
<b>pH 4.6</b>				
Variant GATGAGCG	1.83 (0.06)	$6.2 \times 10^{-8}$	30.18 (1.34)	$4.6 \times 10^{-4}$
Wild type AGGGAATT	0.71 (0.03)		70.3 (5.63)	
<b>pH 7.0</b>				
Variant GATGAGCG	0.56 (0.07)	$8.5 \times 10^{-3}$	71.75 (5.46)	$5.4 \times 10^{-3}$
Wild type AGGGAATT	0.05 (0.01)		134 (3.53)	

$p = 1.9 \times 10^{-3}$ ) (Fig. 3B, Table 1). The variant isoform was similarly more active than the wild type isoform at pH 4.6 (variant  $K_{\text{cat}} = 1.83 \text{ s}^{-1}$ ,  $K_m = 30.18 \mu\text{M}$ ; wild type  $K_{\text{cat}} = 0.71 \text{ s}^{-1}$ ,  $K_m = 70.3 \mu\text{M}$ ,  $p = 6.2 \times 10^{-8}$  and  $4.6 \times 10^{-4}$ , respectively) (Fig. 3C, Table 1). Although enzymatic activity for the variant isoform was considerably lower at pH 7.0 ( $K_{\text{cat}} = 0.56 \text{ s}^{-1}$ ,  $K_m = 71.75 \mu\text{M}$ ), activity was barely detectable for the wild type isoform ( $K_{\text{cat}} = 0.05 \text{ s}^{-1}$ ,  $K_m = 134 \mu\text{M}$ ;  $K_{\text{cat}}$ ,  $p = 8.5 \times 10^{-3}$ ,  $K_m$ ,  $p = 5.4 \times 10^{-3}$ ) (Fig. 3D, Table 1).

**Three-dimensional Modeling of Variant AMCase Isoforms—**The human AMCase crystal structure has recently been published (17). The recombinant protein used to generate this structure contains the variant haplotype amino acid changes. To analyze the consequence of these changes we generated AMCase models by introducing the relevant wild type amino



**FIGURE 4. Structural implications of the three amino acid changes in the variant isoform compared with wild type AMCase.** The wild type AMCase structure is shown in black, the variant isoform structure in red. Only the side chains of those amino acids that are changed in the variant structure compared with wild type and that are implicated in either binding/catalysis or pH specificity (see text) are depicted as sticks (black, wild type; red, variant isoform). The side chains of the three amino acids that are mutated in the variant isoform are also indicated (Asn/Asp-45, Asp/Asn-47, and Arg/Met-61).

acids (see “Experimental Procedures”). Subsequent energy minimization resulted in two models that were used to analyze the effects of these amino acid changes. Structural comparison indicated that the overall protein fold is not altered as indicated by the low root mean square deviation ( $0.113 \text{ \AA}$ ) of the backbone  $C\alpha$  atoms.

According to Olland and co-workers (17), a triad of polar residues (His-208, His-269, and Arg-145) control pH specificity, whereas residues Trp-99, Asn-100, Asp-138, Glu-140, Tyr-212, Asp-213, and Trp-360 are implicated in interaction with the substrate and catalysis. Superpositioning of the two energy-minimized models indicates slight structural changes for some of these amino acids (Fig. 4). For example, His-269 and Arg-145 show a small change in position, whereas the position of His-208 is not changed. Because these residues are reported to play a role in pH specificity, one could envision that a positional rearrangement of these residues alter the pH specificity of the enzyme (17). In the variant haplotype, His-269 forms a hydrogen bond with Asp-213 and thereby influences the  $pK_a$  value of the Asp-138/Glu-140 system (17). A slight change in the position of the His-269 (rotation) in the wild type structure renders this hydrogen bonding less efficient, thereby changing the  $pK_a$  of the Asp-138/Glu-140 system. Moreover, Arg-145 also influences the  $pK_a$  of the Asp-138/Glu-140 system, by hydrogen bonding with the active site residues in a manner similar to His-269. Structural changes of Arg-145 will also affect this interaction.

Besides these positional changes in the triad of polar residues, the positions of some of the residues interacting with the substrate are slightly changed (Trp-99, Asn-100, and Glu-140, see Fig. 4) also, whereas the positions of Asp-138, Tyr-212, Asp-213, and Trp-360 do not change significantly. Changes in the positions of these amino acids might influence the substrate binding and/or stabilization of the transition state intermediate. Altogether these structural changes support the observed

TABLE 2

## Allelic test of association between AMCase SNPs and asthma disease status among African American subjects

Allele frequencies reported are for the second allele listed. Odds ratios (OR), 95% confidence intervals (95% C.I.), and *p* values (*p*) were generated from logistic regression models adjusting for age and gender (and % ancestry among SAGE and CHIRAH subjects). The CHIRAH and ACRN analysis used the SAGE controls.

SNP	Allele frequency				OR (95% C.I.)	<i>p</i>
	SAGE cases ( <i>n</i> = 264)	CHIRAH cases ( <i>n</i> = 321)	ACRN cases ( <i>n</i> = 334)	SAGE controls ( <i>n</i> = 176)		
			%			
G339T	16.8	16.7	18.2	25.9	0.57 (0.38–0.83) <sup>a</sup> 0.62 (0.43–0.89) <sup>b</sup> 0.64 (0.47–0.88) <sup>c</sup>	0.004 0.01 0.005
G461A	17.1			14.5	1.15 (0.74–1.78)	0.525
A531G	4			4.6	0.90 (0.42–1.90)	0.774
G1172A	22.4			20.7	1.25 (0.85–1.83)	0.255
T1218C	32			39.2	0.81 (0.58–1.13)	0.216
G1452T	23.2			21.7	1.26 (0.86–1.84)	0.23

<sup>a</sup> Results for SAGE analysis.

<sup>b</sup> Results for CHIRAH analysis.

<sup>c</sup> Results for ACRN analysis.

differences in pH specificity and the overall alteration in catalytic activity of the variant human AMCase isoform.

**Asthma Association Analysis of AMCase SNPs**—Based on the large gain in enzymatic activity conferred by the variant genetic isoform of the AMCase protein, we hypothesized that the SNP tagging the haplotype, which encoded this variant AMCase protein (G339T), may be associated with asthma. We genotyped this and the other five non-synonymous AMCase SNPs (G461A, A531G, G1172A, T1218G, G1452T) in the SAGE study subjects (Table S7). The SAGE study includes 264 asthmatic cases and 176 healthy controls all self-identified as African Americans. Allele frequencies in SAGE cases and controls are displayed in Table 2. Using logistic regression the only SNP to achieve statistically significant association with asthma disease status was the SNP tagging the gain-of-function AMCase haplotype, G339T (Table 2). The minor T allele of SNP G339T was present at a frequency of 25.9% in the controls and 16.8% in asthmatic cases, revealing an association with asthma protection (odds ratio = 0.57, 95% confidence interval = 0.38–0.83, *p* = 0.004). To confirm the lower allele frequency of SNP G339T among asthmatics we genotyped two additional independent groups of African American asthmatic cases. In African Americans with asthma from the CHIRAH study (*n* = 321) and ACRN study (*n* = 334) we observed allele frequencies of 16.7 and 18.2%, respectively, nearly identical to that of SAGE asthmatic cases (Table S7). Comparing allele frequencies of the G339T SNP in CHIRAH and ACRN asthmatic cases to SAGE controls by logistic regression resulted in statistically significant associations with asthma protection among both studies (0.62 (0.43–0.89), *p* = 0.01; 0.64 (0.47–0.88), *p* = 0.005), respectively (Table 2). We also tested the same 6 SNPs screened among African Americans for association with asthma in the GALA family based trios study (trios *n* = 700) by transmission disequilibrium test (Table S7). The GALA study includes both foreign and United States resident Mexicans and Puerto Ricans recruited to allow determination of whether genetic effect modification occurs by ethnicity or immigration status. Analyzing the GALA cohort stratified by ethnicity the only significant association was with SNP A531G (*p* = 0.03) among Puerto Ricans, but not Mexicans (see Table S9). We did not observe a significant association between SNP G339T and asthma in either Mexican or Puerto Rican trios. However, examining the cohort stratified by immigration status we found significant

undertransmission (indicating protective status) of the 339 T allele among Latino Americans (*p* = 0.04), but not foreign Latinos (*p* = 0.94). These results replicate both the presence and direction of the SNP G339T association observed in African Americans, as the 339 T allele is observed to be protective in both United States resident populations. To further explore the broader applicability of our association between the gain-of-function AMCase haplotype and asthma protection we tested the G339T tagging SNP in an additional asthmatic case-control group of African American (*n* = 259) and Latino (*n* = 476) children recruited as part of the Children's Health Study from 12 Southern California communities (21–23). The G339T SNP was associated with asthma protection among Latino subjects (*p* = 0.02), replicating the genetic association observed among the GALA study Latinos. However, the G339T SNP was not associated among the African American subjects from the CHS cohort. In total our genetic results indicate that the gain-of-function AMCase haplotype is associated with asthma protection in some populations.

## DISCUSSION

The enzymatic activity of AMCase has been shown to be critical in the regulation of pulmonary Th2 inflammation in both murine models exposed and unexposed to polymeric chitin. Based on the importance of AMCase enzymatic activity in these processes we have explored the role of common coding polymorphisms in the human AMCase gene on both enzyme structure and function with subsequent determination of their effect on asthma disease risk. Our comprehensive resequencing of all coding portions of the AMCase gene has revealed the highly polymorphic nature of the gene, as we found 8 amino acid changing (non-synonymous) variants with a frequency of at least 5% in multiple United States ethnic populations. Through haplotype analysis we determined that these SNPs associate to form 6 common gene-wide AMCase haplotypes that code for the different AMCase protein isoforms. One of these haplotypes contained the variant alleles of three linked SNPs located in close proximity to the catalytic site of the AMCase protein. Biochemical comparison of the variant isoform (containing variants in close proximity to the active site) to the wild type isoform revealed the former to confer faster kinetics of chitin cleavage and greater retention of enzymatic activity over a wider range of pH values. These variant amino



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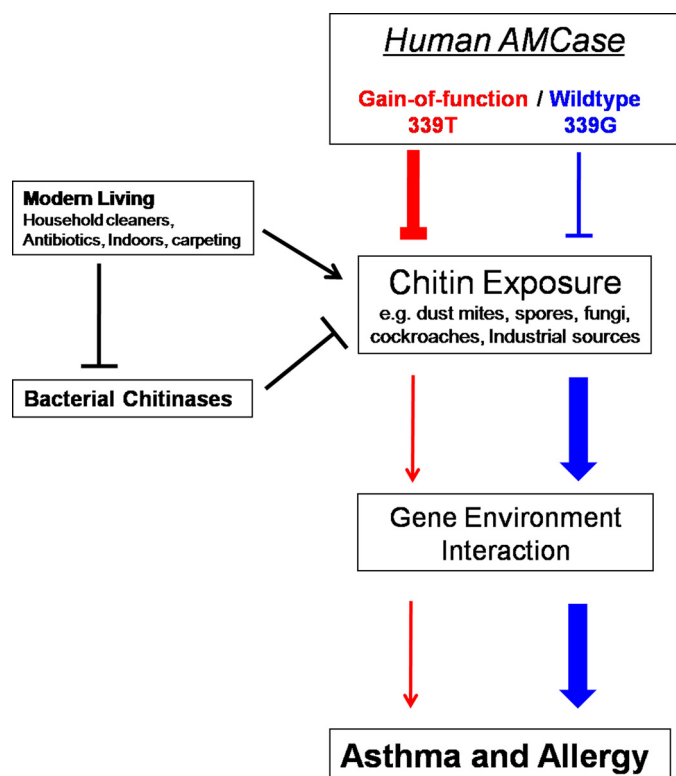
acids were found to alter the positioning of several key amino acids involved in both pH specificity and substrate binding. Interestingly, we found significant associations between the 339 T allele, which tags the variant isoform and protection from asthma in three of four African American and both Latino American study populations examined. These results provide the first evidence that differential AMCase enzymatic activity may modulate risk for human asthma.

This study represents the first biochemical and structural analysis of the AMCase enzyme accounting for genetic variation in the *AMCase* gene. Although we only tested one of the five wild type (with respect to the three linked variants tagged by G339T) AMCase isoforms, the haplotype encoding this isoform is found at a high frequency in Mexicans (63.9%), Puerto Ricans (44.1%), and African Americans (18%). The initial biochemical analysis of human AMCase performed by Chou *et al.* (7) reported a  $K_{\text{cat}}$  of  $0.81 \text{ s}^{-1}$  and a  $K_m$  of  $61 \mu\text{M}$ , which are similar to the values we report for the wild type AMCase isoform ( $K_{\text{cat}} = 0.71 \text{ s}^{-1}$ ,  $K_m = 70.3 \mu\text{M}$ ). In contrast we observed significantly faster kinetic parameters for the variant AMCase isoform ( $K_{\text{cat}} = 1.83 \text{ s}^{-1}$ ,  $K_m = 31 \mu\text{M}$ ), giving further credence to the anomalous nature of the variant haplotype tested and the representative nature of the wild type haplotype tested. These results are supported by our modeling of the AMCase isoforms, which reveals structural changes in three of the seven substrate interacting amino acids (Trp-99, Asn-100, and Glu-140), implying stabilization of the substrate may be responsible for the observed differences in activity. Additionally, pH profile results of Chou *et al.* (7) mirrored those determined for the wild type isoform tested and differed dramatically from the variant isoform at acidic and neutral pH values. Likewise, we found hydrogen bonding between 2 of 3 of the polar residues critical for AMCase pH specificity and active site residues to be less efficient, resulting in changes in the  $\text{p}K_a$  of the Asp-138/Glu-140 system. These structural changes in the enzyme could be responsible for both the isoform differences in pH specificity and enzyme kinetics. It is interesting to note that the variant alleles that define the variant isoform, A290G/G296A/G339T, match those nucleotides reported at the corresponding position in the murine *Amcase* gene. Similar to the variant human isoform, murine *Amcase* exhibits excellent stability and kinetic activity at low pH values, supporting the role of these variants in stabilizing the variant human AMCase isoform at low pH values (24).

Our genetic results represent the first screen of *AMCase* genetic variation in Latino Americans or African Americans, two groups with high asthma prevalence and morbidity. The two prior human genetic studies of *AMCase*, in Indian and German cohorts, both found significant associations between *AMCase* SNPs and asthma disease status, increasing our confidence that AMCase participates in human asthma pathogenesis. This study is the first to screen the full-length *AMCase* gene (AF290004), whereas prior studies were based on a shorter version of the *AMCase* transcript, lacking the fourth exon, which is required for enzymatic activity. This fourth exon contains the novel SNPs in the gain-of-function AMCase isoform that was associated with asthma protection in both African Americans and Latino Americans. To determine the broader applicability

of our genetic findings and increase confidence that our results in the SAGE and GALA cohorts were not spurious findings we attempted replication in multiple other genetic study groups. The 339 T asthma case allele frequency in African Americans was confirmed in two independent asthma case groups. Additionally, the association with SNP G339T in the GALA Latino Americans was replicated in the Latino population from CHS. However, we did not observe significant associations with asthma and SNP G339T in the African American population from CHS. This result may be a spurious finding based on the small size of this study group ( $n < 300$ ) or could represent differences in environmental effect modifiers in Southern *versus* Northern California. Further genetic studies will be needed to confirm our results in African Americans. However, our results are reinforced by the fact that the direction of association was consistent across both ethnicities, multiple independent studies, and different study designs (case-control *versus* family based trios). Future studies could likely provide more insight with the inclusion of environmental factors into modeling, most notably environmental chitin exposure, which was shown to be proinflammatory in the lungs of mice.

The dramatic increases in chitinase activity of the variant AMCase isoform at pH 2.2 (4-fold higher) and 7.0 (10-fold higher), corresponding to the pH of the lungs and stomach, respectively, is especially relevant because the lumen of these organs are exposed to exogenous chitin through inhalation and ingestion. Indeed, some of the most common indoor allergens in Western societies, such as the United States, include dust mites, molds, and cockroaches, all chitin-containing organisms. In fact, dust mite and cockroach feces, components of house dust and a major source of allergy, are surrounded by a chitinous peritrophic membrane (25, 26). Lack of childhood exposure to bacterial products such as endotoxin has been associated with risk of asthma in some studies, supporting the hygiene hypothesis of allergic diseases. In this context, it is noteworthy that chitinases produced by bacteria comprise an important mechanism for degradation and turnover of the massive amounts of chitin produced in marine environments (27). The widespread use of anti-microbial cleaning products and antibiotics in modern Western society may have inadvertently contributed to the accumulation of environmental chitin through suppression of bacterial populations producing chitinases critical to environmental chitin degradation. Interestingly, individuals employed in industries where exposure to chitin would be anticipated to be high, such as shellfish processors, have a high prevalence of new onset asthma (28). The development of asthma in these industries has been linked with processing steps involving high pressure shell meat removal, which produce vapors and particulate aerosols (28). In settings of high environmental chitin burden it would prove beneficial for subjects to produce a hyperfunctional AMCase enzyme capable of degrading this inflammatory stimulus (see Fig. 5). In this context we note our prior finding that chitotriosidase not AMCase was the primary enzyme responsible for chitinase activity in the bronchoalveolar lavage of asthmatics, smokers, and healthy controls. In this work we found AMCase protein to be overexpressed in asthmatic subjects, which is consistent with our observations here, but the protein was expressed from



**FIGURE 5. Potential causal model describing the interaction of proinflammatory environmental chitin exposures with allergic antigens and the genetic regulation of this process by chitinases.** In line with the hygiene hypothesis we predict decreased bacterial populations (and accompanying bacterial chitinases) due to the prevalence of anti-microbial products in developed societies, which may increase environmental chitin exposure. Moreover, in this environment of increased chitin exposures a gain-of-function AMCase variant may be protective against development of allergy and asthma.

a splice variant of the *AMCase* gene resulting in a non-functional protein. Differential splicing of the *AMCase* RNA may be dependent on physiologic conditions within the lung. For example, the asthmatic subjects in this study were all stable and had mild disease at the time of sampling. Because *AMCase* expression is strongly regulated by active Th2 inflammation it is very possible the active isoform predominates in severe asthmatics and/or during asthma exacerbations. Furthermore, consistent with the disease model presented here, expression of the active isoform could be acutely up-regulated by environmental chitin exposures.

Intriguingly, the enhanced acidic pH activity of the protective isoform of *AMCase* may reflect improved activity in the stomach, where the degradation of ingested polymeric environmental chitin or chitin-containing microorganisms could lead to modifications of the bowel commensal flora or to alterations in immune responses to ingested allergens. Ingested polymeric chitin has been observed to disrupt interactions with host proteins involved in regulating bacterial adherence to the gastrointestinal epithelium, such as RegIII $\gamma$ , and to be used as the preferred energy source by certain gut bacteria (29, 30). Alterations in intestinal microflora have been observed to modify the subsequent immune response to allergens in the lung in experimental models, supporting this possibility (31).

In summary, we have identified a common genetic variant in

the *AMCase* gene, which results in an *AMCase* isoform that displays significant enzymatic activity over a much wider pH range, and exhibits dramatically enhanced kinetics of chitin cleavage at all pH values relevant to *AMCase* expression in the human body. In line with the observed differences in enzymatic activity we found the three-dimensional model of the variant enzyme to exhibit significant rotation in amino acid residues critical for enzyme function. Finally, we find the haplotype encoding this isoform to be associated with protection from asthma in several United States ethnic populations. These results raise the possibility that increased *AMCase* enzymatic activity could be protective against the development of human asthma, possibly through cleavage of inflammatory chitin polymers. Further studies examining the population effects on asthma and allergy of high environmental chitin exposure are warranted to explore this hypothesis.

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