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# Identification of a Novel Acidic Mammalian Chitinase Distinct from Chitotriosidase*

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**Rolf G. Boot‡§, Edward F. C. Blommaart‡, Erwin Swart‡, Karen Ghauharali-van der Vlugt‡, Nora Bijl‡, Cassandra Moe¶‖, Allen Place¶‖, and Johannes M. F. G. Aerts‡**

*From the ‡Department of Biochemistry, University of Amsterdam, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands and the ¶Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202*

**Chitinases are ubiquitous chitin-fragmenting hydrolases. Recently we discovered the first human chitinase, named chitotriosidase, that is specifically expressed by phagocytes. We here report the identification, purification, and subsequent cloning of a second mammalian chitinase. This enzyme is characterized by an acidic isoelectric point and therefore named acidic mammalian chitinase (AMCase). In rodents and man the enzyme is relatively abundant in the gastrointestinal tract and is found to a lesser extent in the lung. Like chitotriosidase, AMCase is synthesized as a 50-kDa protein containing a 39-kDa N-terminal catalytic domain, a hinge region, and a C-terminal chitin-binding domain. In contrast to chitotriosidase, the enzyme is extremely acid stable and shows a distinct second pH optimum around pH 2. AMCase is capable of cleaving artificial chitin-like substrates as well as crab shell chitin and chitin as present in the fungal cell wall. Our study has revealed the existence of a chitinolytic enzyme in the gastrointestinal tract and lung that may play a role in digestion and/or defense.**

Next to cellulose, chitin is the most abundant glycopolymer on earth, being present as a structural component in coatings of many species, such as the cell wall of most fungi (1), the microfilarial sheath of parasitic nematodes (2, 3), and the exoskeleton of all types of arthropods (4), and in the lining of guts of many insects (5). Chitinases (EC 3.2.1.14) are endo-$\beta$-1,4-*N*-acetylglucosaminidases that can fragment chitin and have been identified in several organisms (6). Until a few years ago it was generally assumed that man lacks the ability to produce a functional chitinase. Our observation of a markedly elevated chitotriosidase activity in plasma of symptomatic Gaucher patients formed the basis for the subsequent identification of a human phagocyte-specific chitinase, named chitotriosidase (7–9). Tissue macrophages can synthesize large amounts of chitotriosidase upon an appropriate stimulus, such as the massive lysosomal lipid accumulation that occurs in macrophages of Gaucher patients (7). Chitotriosidase is largely secreted as a 50-kDa active enzyme containing a C-terminal chitin binding domain (10, 11). In macrophages some enzyme is proteolytically processed to a C-terminally truncated 39-kDa form with hydrolase activity that accumulates in lysosomes of these cells (10). The 50-kDa chitotriosidase form is also synthesized by progenitors of neutrophilic granulocytes (9) and stored in their specific granules (9, 12).

Chitotriosidase is remarkably homologous to chitinases from plants, bacteria, fungi, nematodes and insects (8, 9). Analogous to some plant chitinases, recombinant chitotriosidase has been found to inhibit hyphal growth of chitin-containing fungi such as *Candida* and *Aspergillus* species.[1] The specific expression by phagocytes also suggests a physiological role in defense against chitin-containing pathogens.

A recessively inherited deficiency in chitotriosidase activity is frequently encountered (7, 13). About 1 in 20 individuals is completely deficient in enzymatically active chitotriosidase, because of a 24-base pair duplication in the chitotriosidase gene (14). This duplication, which occurs panethnically, leads to strongly reduced amounts of an abnormally spliced mRNA only, encoding an enzymatically inactive protein that lacks an internal stretch of 29 amino acids (14). In Caucasian populations, up to 35% of all individuals carry this abnormal chitotriosidase allele and about 5% are homozygous for this allele (14). The prevalence of deficiency suggests that chitotriosidase no longer fulfills an important defense function under normal circumstances or, alternatively, that other mechanisms may compensate the lack of functional chitotriosidase.

To test whether compensatory mechanisms exist, we have searched for other chitinases in mammals. The discovery of a second mammalian chitinolytic enzyme is described here. The properties of this acidic mammalian chitinase (AMCase)[2] are reported, and the possible implications of its existence are discussed.

## EXPERIMENTAL PROCEDURES

*Enzyme Assays*—Chitinase enzyme activity was determined with the fluorogenic substrates 4-methylumbelliferyl $\beta$-D-*N*,*N*′-diacetylchitobiose (4MU-chitobiose; Sigma) and 4-methylumbelliferyl $\beta$-D-*N*,*N*′,*N*″-triacetylchitotriose (Sigma). Assay mixtures contained 0.027 mM substrate and 1 mg/ml of bovine serum albumin in McIlvaine buffer (100 mM citric acid, 200 mM sodium phosphate) at the indicated pH. The standard enzyme activity assay for human chitotriosidase with 4-methylumbelliferyl $\beta$-D-*N*,*N*′,*N*″-triacetylchitotriose substrate was performed at pH 5.2, as previously described (7). The standard AMCase

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§ To whom correspondence should be addressed: Dept. of Biochemistry, University of Amsterdam, Academic Medical Center, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands. Tel.: 31-20-5665161; Fax: 31-20-6915519; E-mail: r.g.boot@amc.uva.nl.

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[1] R. G. Boot, E. F. C. Blommaart, G. W. Gooday, B. A. Friedman, and J. M. F. G. Aerts, manuscript in preparation.

[2] The abbreviations used are: AMCase, acidic mammalian chitinase; 4MU-chitobiose, 4-methylumbelliferyl $\beta$-D-*N*,*N*′-diacetylchitobiose; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag; PCR, polymerase chain reaction.

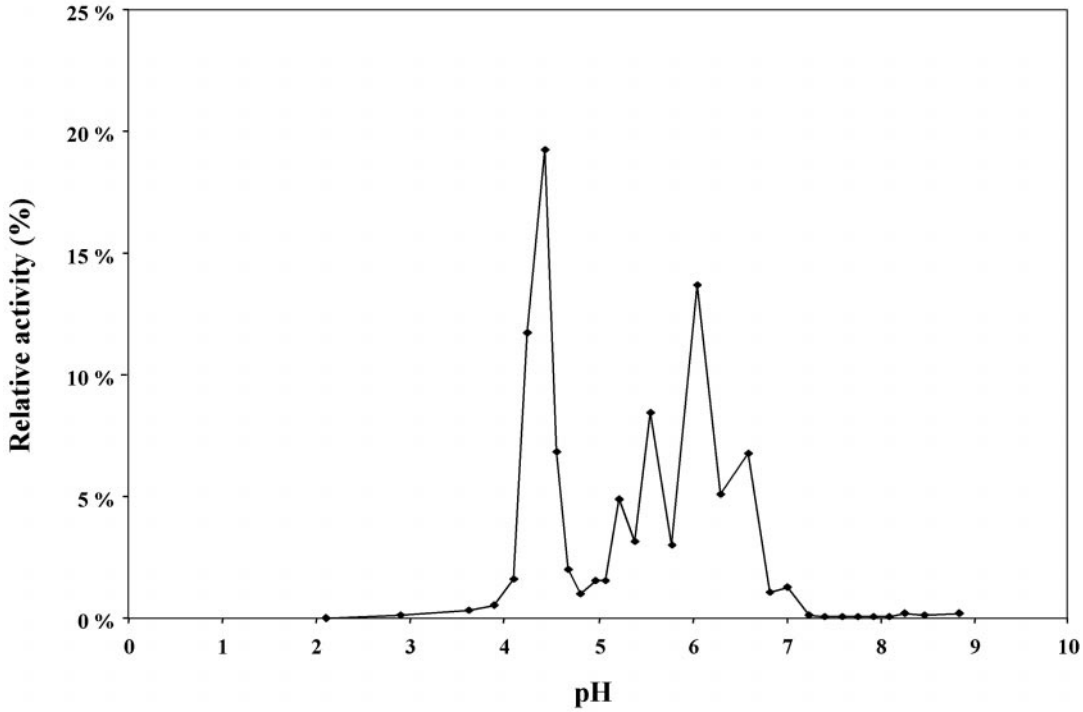



FIG. 1. **Isoelectric focusing profile of chitinolytic activity in mouse lung extract.** Isoelectric focusing was performed as described under "Experimental Procedures." Chitinolytic activity was measured using 4MU-chitotrioside substrate. The enzyme activity present in the different isoelectric focusing fractions is expressed as a percentage of the total activity present in all fractions.

enzyme activity assays with 4MU-chitobiose substrate were performed at pH 4.5.

Crab shell chitin (Poly- (1–4)-β-D-*N*-acetylglucosamine, Sigma) was used as a natural substrate to determine chitinase activity as described (10). The chitin fragments were analyzed by fluorophore-assisted carbohydrate electrophoresis as described by Jackson (15).

*Degradation of Fungal Cell Wall Chitin*—Measurements of chitin formation during regeneration of fungal spheroplasts was performed as described by Hector and Braun (16). Briefly, spheroplasts were prepared from the *Candida albicans* strain CAi-4 (*ura3*), grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28 °C. Cells were concentrated by centrifugation and incubated with 2.5 mg/ml zymolyase (100T, ICN Immuno Biologicals, Costa Mesa, CA) in buffer containing 50 mM sodium phosphate, pH 7.5, 1.2 M sorbitol, and 27 mM β-mercaptoethanol for 60 min at 37 °C. After extensive washing, spheroplasts were allowed to regenerate in 96-well microtiter plates in regeneration buffer (0.25% (w/v) MES buffer, pH 6.7, containing 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate; Sigma), 0.15% (w/v) ammonium sulfate, 2% (w/v) glucose, 1.2 M sorbitol, 20 μg/ml uridine) at 37 °C. Chitinase enzyme preparations were added to a final concentration of 3 μg/ml. After a 2-h incubation, 50 μl of 300 μg/ml Calcofluor white (Sigma) in 10 mM sodium phosphate buffer, pH 7.5, containing 1.2 M sorbitol was added. After 5 min the plates were washed with buffer only, and fluorescence was determined using a LS 50 Perkin Elmer fluorimeter (excitation, 405 nm; emission, 450 nm).

*Purification of the Mouse AMCase*—Detergent-free extracts of mouse tissues were prepared by homogenization in 10 volumes of potassium phosphate buffer, pH 6.5, using an Ultra-turrax and centrifugation for 20 min at 15,000 × *g*. The mouse intestine extract was adjusted to pH 5.0 by the addition of citric acid (0.2 M); NaCl was added to a final concentration of 2 M. A chitin column was prepared by mixing 10 g of swollen Sepharose G25 fine (Amersham Pharmacia Biotech) with 300 mg of colloidal chitin, followed by equilibration with phosphate-buffered saline containing 2 M NaCl. The extracts were applied onto the column with a flow speed of 0.4 ml/min. After extensive washing, bound chitinase was eluted from the column with 8 M urea, which was subsequently removed by dialysis. Protein concentrations were determined according to the method of Lowry *et al.* (17) using bovine serum albumin as a standard. Fractions containing chitinase activity were subjected to SDS-PAGE and Western blotting as described (8). N-terminal protein sequencing was performed as described using a Procise 494 sequencer (Applied Biosystems Perkin Elmer) (8). Colloidal chitin was prepared as described by Shimahara and Takiguchi (18).

*SDS-PAGE and Glycol-Chitin Gel Electrophoresis*—SDS-PAGE was performed with a Amersham Pharmacia Biotech phast gel system, according to the instructions of the manufacturer, using 12.5% polyacrylamide gels, followed by silver staining. Glycol-chitin electrophoresis was conducted as described by Escott and Adams (19), except for an extension of the renaturation time to 8 h. Glycol-chitin was prepared from glycol chitosan (Sigma) as described by Trudel and Asselin (20).

*Isoelectric Focusing*—The native isoelectric point of chitinases was determined by flat bed isoelectric focusing in granulated Ultrodex gels (Amersham Pharmacia Biotech) as described (8).

*Northern Blot and RNA Master Blot Analysis*—Total RNA was isolated using RNAzol B (Biosolve, Barneveld, The Netherlands) according to the instructions of the manufacturer. Northern blots, using 15 μg of total RNA, were performed as described (9). Human and mouse RNA Master Blots (CLONTECH, Palo Alto, CA) were used to examine the tissue distribution of transcripts according to the instructions of the manufacturer. The following probes were used: the full-length mouse acidic chitinase cDNA, the human EST clone oq35c04.s1 (GenBank™ accession number AA976830) and glyceraldehyde-3-phosphate dehydrogenase as control. Radiolabeling and hybridization was conducted as described previously (9). Quantification of radioactivity was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

*cDNA Cloning of the Mouse AMCase*—Reverse transcription polymerase chain reaction (PCR) fragments were generated from mouse lung total RNA using degenerate oligonucleotides, as described (9). Obtained fragments were cloned in pGEM-T (Promega, Madison, WI), sequenced, and compared with the amino acid sequence established by N-terminal protein sequencing. A comparison with the GenBank™ mouse EST (expressed sequence tag) data base using the Basic local alignment search tool (BLAST) at the National Center for Biotechnology Information showed that several EST clones matched the mouse chitinase cDNA sequence, for example, ms33 h09.y1 (GenBank™ accession number AI892792). This clone was obtained and sequenced. Antisense primers were generated complementary to the most 3′ region of the EST clone (A tail primer, 5′-TTTTGGCTACCAATTTTATTGC-3′) and two internal antisense primers (MAS1, 5′-CAGCTACAGCAGCAGTAACCATC-3′ and MAS2, 5′-TTCAGGGATCTCATAGCCAGC-3′). The MAS1 and MAS2 primers were used to clone the most 5′ end of the mouse acidic chitinase cDNA using 5′ rapid amplification of cDNA ends and the Marathon-Ready mouse Lung cDNA kit (CLONTECH) according to the instructions of the manufacturer. To obtain the complete coding sequence a 5′ sense primer was generated (MS1, 5′-CGATGGCCAAGCTACTTCTCGT-3′). The total cDNA sequence was subsequently

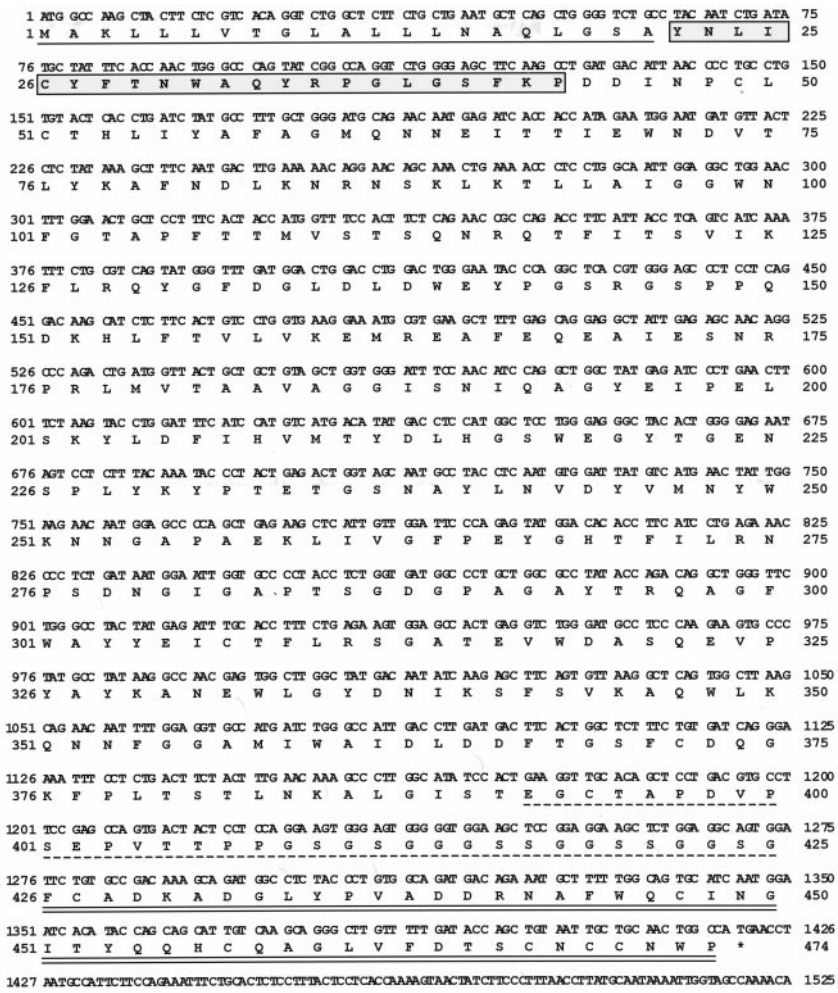


FIG. 2. **Mouse AMCase cDNA sequence and deduced amino acid sequence.** The cDNA sequence (GenBank™ accession number AF290003) is indicated by the *upper sequence*, and the deduced amino acid sequence is depicted *below* the nucleotide sequence. The characteristic hydrophobic signal peptide (amino acids 1–21) is *underlined* with a *single line*. The putative chitin binding domain (amino acids 426–473) is *underlined* with a *double line*. The hinge region separating the catalytic domain from the chitin binding domain is *underlined* with a *dashed line*. The part of the protein purified from mouse intestine that was determined by Edman sequencing is *boxed*.

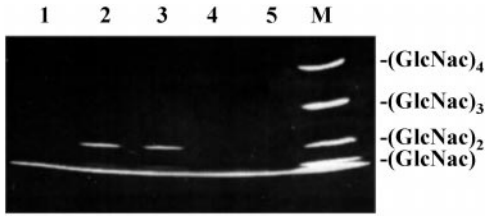



FIG. 3. **Degradation products with colloidal chitin as substrate.** The fluorophore-assisted carbohydrate electrophoresis technique (described under "Experimental Procedures") was used to visualize the cleavage products of recombinant human chitotriosidase and recombinant mouse AMCase using colloidal chitin as substrate. *Lane 1*, no enzyme added. *Lane 2*, products formed after incubation with 50-kDa recombinant human chitotriosidase and chitin. *Lane 3*, products formed with recombinant mouse AMCase and chitin. *Lane 4*, human chitotriosidase incubated without substrate. *Lane 5*, mouse AMCase incubated without substrate. Marker lane is indicated with *M* (sugar polymers are indicated on the *right-hand side*).

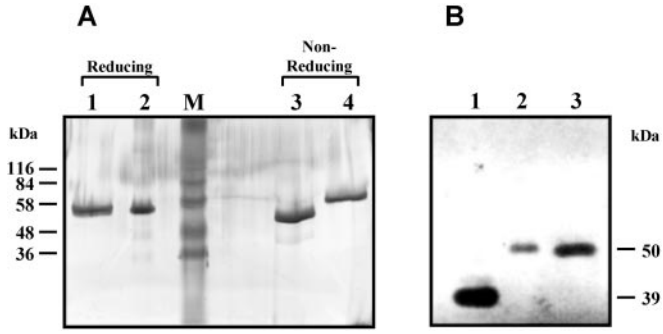
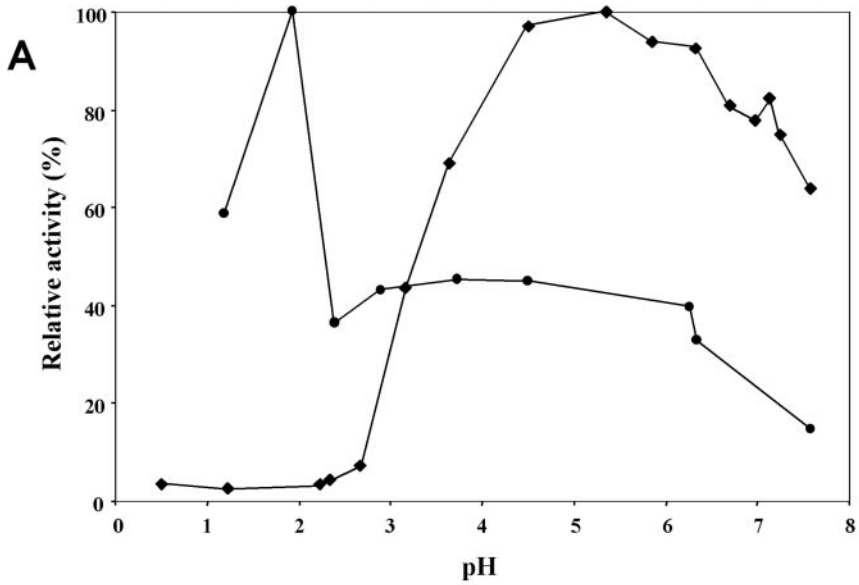



FIG. 4. **Electrophoretic behavior of chitinases.** *A*, purified recombinant human chitotriosidase and mouse AMCase were separated on a 12.5% SDS-PAGE gel in the presence or absence of a reducing agent and visualized by silver staining as described under "Experimental Procedures". *Lane 1*, recombinant mouse AMCase under reducing conditions. *Lane 2*, recombinant human chitotriosidase under reducing conditions. *Lane 3*, recombinant human chitotriosidase under nonreducing conditions. *Lane 4*, recombinant mouse AMCase under nonreducing conditions. *M* indicates the molecular mass standards (mass (kDa) indicated at the *left-hand side*). *B*, the same purified recombinant enzymes as described in *A* were separated on a 10% SDS-PAGE gel containing glycol-chitin as described under "Experimental Procedures." Chitinolytic activity was visualized as clearing zones in the gel. *Lane 1*, recombinant human 39-kDa chitotriosidase. *Lane 2*, recombinant human 50-kDa chitotriosidase. *Lane 3*, recombinant mouse AMCase (mass (kDa) indicated at the *right-hand side*).

generated using MS1 and the A tail primer. The fragments of two independent PCRs were cloned into pGEM-T (Promega), and the nucleotide sequences of two independent clones from each PCR were sequenced from both strands by the procedure of Sanger using fluorescent nucleotides on an Applied Biosystems 377A automated DNA sequencer following Applied Biosystems protocols.

*cDNA Cloning of the Human AMCase*—Comparison of the mouse AMCase cDNA sequence with the human EST data base (National




**B**

| | pH2 | pH7 |
|---|---|---|
| h-chitotriosidase | 0% | 100% |
| m-AMCase | 108% | 98% |

**C**

| TCA (%) | 0.5 | 1.25 | 2.5 | 5.0 |
|---|---|---|---|---|
| h-chitotriosidase | 58% | 74% | 97% | 100% |
| m-AMCase | 0% | 8% | 74% | 100% |

FIG. 5. **Effects of acidic pH.** *A*, pH activity profile of the different chitinases. The pH optima were determined by monitoring enzyme activity at the indicated pH in McIlvaine buffer. ♦, purified human recombinant chitotriosidase; ●, purified mouse AMCase. *B*, effects of acidic preincubation. Purified recombinant human chitotriosidase and mouse AMCase were preincubated for 30 min at the indicated pH in McIlvaine buffer prior to enzyme activity measurement at the assay pH (see "Experimental Procedures"). Activity prior to incubation at the indicated pH is defined as 100%. *C*, precipitation by trichloroacetic acid. Purified recombinant human chitotriosidase and mouse AMCase were incubated with the indicated percentages of trichloroacetic acid (*TCA*). The amount of enzyme activity Precipitated is shown as percentage of initial amounts.

Center for Biotechnology Information) revealed the presence of a human EST clone oq35c04.s1 (GenBank™ accession number AA976830) highly homologous to the mouse acidic chitinase. Following the same strategy, the full-length human AMCase cDNA was cloned using human stomach total RNA (CLONTECH) for the reverse transcription PCR with the same degenerate primers. A human Marathon-Ready Lung cDNA was used to clone the most 5′ end of the cDNA by 5′ rapid amplification of cDNA ends using the following primers: HAS2 (5′-TCTGACAGCACAGAATCCACTGCC-3′) and HAS3-A tail (5′-TTGACTGCTGATTTTATTGCAG-3′). The total cDNA sequence was subsequently generated using HS1 (5′-GCTTTCCAGTCTGGTGGTGAAT-3′) and HAS3-A tail. The fragments of two independent PCRs were cloned in pGEM-T (Promega) and sequenced as described above.

*Transient Expression in COS-1 Cells*—Transient expression of the various cDNAs in COS-1 cells was performed exactly as described previously (9).

## RESULTS

To obtain more insight into the potential occurrence of multiple mammalian chitinases, tissues of mouse and rat were examined for chitinolyic activity using the chitin-like 4-methylumbelliferyl-β-chito-oligosaccharide substrates. In extracts of stomach and intestine, a high level of activity was detected, whereas extracts of lung, tongue, kidney, and plasma showed significant but lower activities. Isoelectric focusing of a mouse lung extract revealed a major peak of chitinolytic activity with pI of 4.5, whereas minor peaks were found with pI levels of 5.5–6.5 (Fig. 1). Extracts of other mouse and rat tissues showed similar profiles of chitinolytic activity upon isoelectric focusing. The observed rodent chitinase with acidic isoelectric point (pI 4.5 form) differs strikingly from human chitotriosidase which has an apparent neutral/basic pI.

The mouse acidic chitinase activity was found to bind to chitin particles with high affinity. Chitin affinity chromatography was used to purify the enzyme, as described under "Experimental Procedures." The procedure resulted in a 30,000-fold purification of an apparently homogeneous 50-kDa protein. The specific activity of the purified enzyme was 3.9 nmol of 4-methylumbelliferyl-chitotrioside hydrolyzed per mg per hour at pH 5.2, which is almost identical to that of human chitotriosidase.

The N-terminal amino acid sequence of purified acidic chitinase was determined (Fig. 2) and was found to be almost identical to that of other known members of the chitinase family. This amino acid sequence allowed the cloning of the corresponding full-length mouse acidic chitinase cDNA, as described under "Experimental Procedures." The full-length cDNA predicts the synthesis of a 50-kDa (pI 4.85) protein with a characteristic signal peptide (Fig. 2). Expression of this cDNA in COS-1 cells led to the secretion of an 50-kDa active chitinase with a pI of 4.8.

The mouse acidic chitinase protein shows considerable sequence homology to human chitotriosidase. Comparison of the

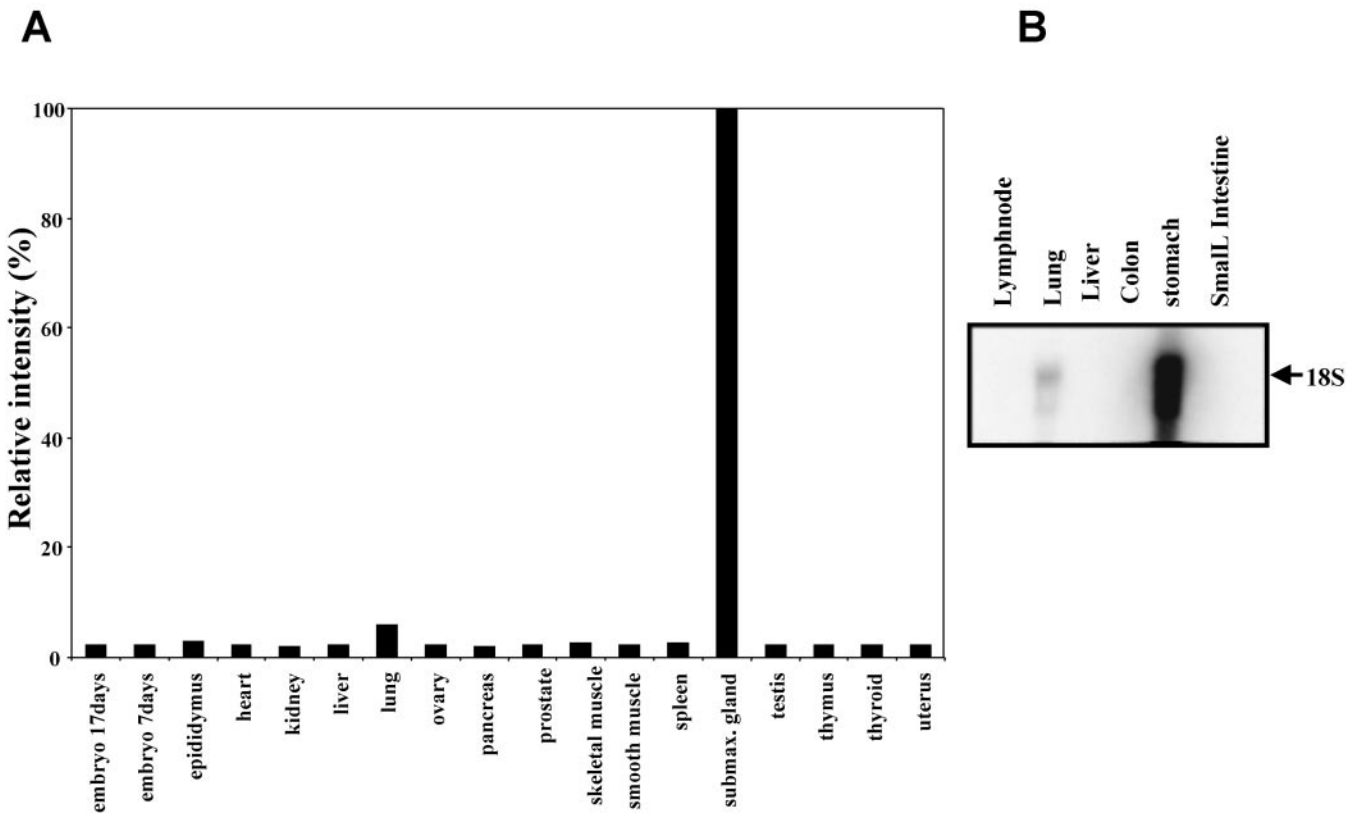



FIG. 6. **Tissue distribution of mouse AMCase mRNA.** *A*, The relative expression levels of mouse AMCase in various mouse tissues as determined by dot blot analysis using a RNA Master Blot (CLONTECH) as described under "Experimental Procedures." The highest level of expression is defined as 100%. *B*, Northern blot of RNA isolated from the indicated mouse tissues. 15 $\mu$g of total RNA was separated on an agarose gel as described under "Experimental Procedures." The full-length mouse AMCase cDNA was used as probe. As a control for RNA loading a glyceraldehyde-3-phophate dehydrogenase probe was used (data not shown). The position of the 18 S ribosomal RNA band is indicated.

amino acid sequence of both mature proteins revealed an identity of 52% and a similarity of 60%. Like the human chitotriosidase, the mouse enzyme is predicted to contain an N-terminal catalytic domain of about 39 kDa, a hinge region, and a C-terminal chitin binding domain (Fig. 2). The mouse acidic chitinase, like chitotriosidase, is predicted to lack *N*-linked oligosaccharides, explaining the observed absence of binding to concanavalin A (data not shown).

Several different assays revealed that the mouse acidic chitinase is able to degrade chitin and therefore has to be considered to be a true chitinase. Firstly, fluorophore-assisted carbohydrate electrophoresis analysis revealed that recombinant mouse chitinase, like chitotriosidase, releases mainly chitobioside fragments from chitin (Fig. 3). Secondly, like chitotriosidase and some other nonmammalian chitinases, the mouse acidic chitinase is strongly inhibited ($IC_{50}$ of 0.4 $\mu$M) by the competitive chitinase inhibitor allosamidin (21–23). Finally, the mouse acidic chitinase and chitotriosidase were both able to digest chitin in the cell wall of regenerating spheroplasts of *C. albicans*. The chitin content of the cell wall was determined with the Calcofluor white stain (see "Experimental Procedures"). When regenerating cells were incubated for 2 h with 3 $\mu$g/ml recombinant chitotriosidase or 3 $\mu$g/ml recombinant mouse acidic chitinase, the chitin content was reduced by 27 and 33%, respectively. Concomitant presence of allosamidin during the incubation completely abolished the effect of both recombinant chitinases.

The apparent molecular masses of identically produced recombinant human chitotriosidase and recombinant mouse acidic chitinase are comparable when run on a SDS-PAGE gel under reducing conditions. However, under nonreducing conditions, the mouse acidic chitinase migrates significantly slower than the human chitotriosidase (Fig. 4*A*). Upon gelelectrophoresis (under nonreducing conditions) in polyacrylamide gels containing glycolchitin, followed by regeneration of active enzyme and detection of the local digestion of glycolchitin using Calcofluor staining, the mouse acidic chitinase migrates slightly faster than human chitotriosidase (Fig. 4*B*).

A further striking difference between human chitotriosidase and the mouse acidic chitinase is their behavior at acidic pH. The mouse acidic chitinase shows a pronounced pH optimum at pH 2.3 and a less pronounced optimum at more neutral pH (pH 4–7). Chitotriosidase, however, shows only a broad pH optimum (Fig. 5*A*) and is completely inactivated by pre-incubation at low pH (Fig. 5*B*). In the presence of 0.5% (w/v) trichloroacetic acid 58% of chitotriosidase is precipitated, whereas under similar circumstances the mouse acidic chitinase remains in solution. At 2.5% (w/v) trichloroacetic acid all chitotriosidase precipitates, whereas 26% of mouse acidic chitinase remains unprecipitated (Fig. 5*C*).

Another major difference between human chitotriosidase and the mouse acidic chitinase is revealed by comparison of RNA expression patterns. Although human chitotriosidase mRNA is mainly found in lymph node, bone marrow, and lung, the mouse acidic chitinase mRNA is predominantly found in stomach, submaxillary gland, and, at a lower level, in the lung (Fig. 6). Surprisingly, no mouse acidic chitinase mRNA can be detected in the small intestine, suggesting that the protein in the intestine is probably derived from the upper parts of the gastrointestinal tract, such as the stomach.

In rat tissues a comparable acidic chitinase was observed. Our findings indicate that the acidic chitinase in rodents is distinct from human chitotriosidase. The discrete enzyme is therefore referred to as acidic mammalian chitinase or AMC-

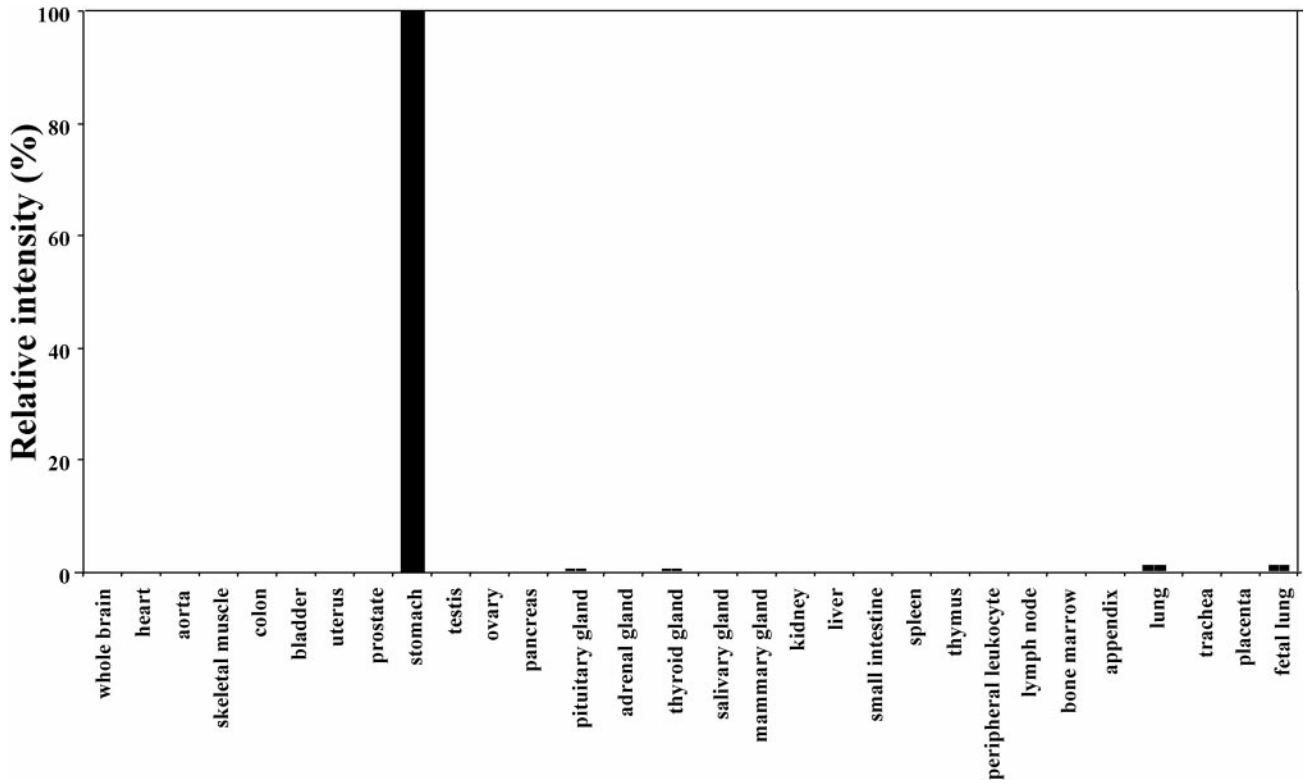



FIG. 7. **Tissue distribution of human AMCase mRNA.** The relative expression levels of human AMCase in various human tissues was determined by dot blot analysis using a RNA Master Blot (CLONTECH) using the oq35c04,s1 EST clone (GenBank™ accession number AA976830) as probe. The highest level of expression is defined as 100%. Several tissues were excluded from the figure because they did not result in detectable signal: amygdala, caudate nucleus, cerebellum, cerebral cortex, frontal lobe, hippocampus, medulla oblongata, occipital lobe, putamen, substantia nigra, temporal lobe, thalamus, nucleus accumbeus, spinal cord, fetal brain, fetal heart, fetal kidney, fetal liver, fetal spleen, and fetal thymus.

ase. It was investigated whether such an acidic chitinase is also present in man. Screening the human EST data base at the National Center for Biotechnology Information with the mouse acidic chitinase cDNA revealed the presence of a highly homologous human EST clone (oq35c04.s1, GenBank™ accession number AA976830). The tissue distribution of this human mRNA was examined using a human Masterblot (CLONTECH). The expression pattern of this mRNA is similar to the expression pattern of the mouse acidic chitinase (Fig. 7), being highly expressed in the stomach and at a lower level in the lung. Using degenerate oligonucleotides directed against members of the chitinase family, we were able to amplify other regions of the human acidic chitinase, generating enough information to clone the full-length human acidic chitinase cDNA (Fig. 8*A*). Screening the GenBank™ data base using the full-length human cDNA revealed that it was almost identical to TSA1902-L (GenBank™ accession number AB025008) and TSA1902-S (GenBank™ accession number AB025009) from a lung cDNA library described by Saito *et al.* (24). These two sequences are most probably splice variants of the human acidic chitinase mRNA. Only expression of full-length human AMCase cDNA in COS-1 cells led to the production of a protein with chitinolytic activity (data not shown). Sequence comparison of the human acidic chitinase and the mouse acidic chitinase revealed an 82% identity and a similarity of 86% (Fig. 8*B*).

The demonstration by Saito *et al.* (24) that the gene encoding TSA1902 is located on chromosome 1p13 indicates that mammals contain indeed at least two discrete genes that encode functional chitinases, being chitotriosidase (locus 1q32) and AMCase (locus 1p13). Definitive proof for the existence of at least two distinct, functional mammalian chitinase genes was recently obtained by the partial cloning of chitotriosidase cDNA from the rat. The cloned rat cDNA (80% of the complete cDNA) encodes a protein that is 80% identical to the human counterpart.

## DISCUSSION

For many years the existence of chitinase has been well documented for a large variety of organisms, including bacteria, plants, insects, and fungi (for an overview see Ref. 6). More recently, it has become clear that mammals also contain such enzymes. Chitotriosidase was the first mammalian chitinase that had been cloned and characterized (7–9). Besides this human phagocyte-specific chitinase, several inactive members of the mammalian chitinase protein family have also been identified. These include oviduct-specific glycoprotein from several mammalian species (reviewed in Refs. 25–27), human HC gp39/YKL-40 (28, 29), mouse BRP39 (30), pig gp38K (31), human YKL-39 (32), and mouse YM1/ECFL/MCRP (33, 34). The functions of these proteins, of which some have been shown to express lectin-like properties (35), are at present unknown. It has been speculated that they might have a role in tissue remodelling processes (28) or chemotaxis (33, 36).

To our knowledge chitotriosidase is the only mammalian chitinase that has been cloned and characterized in detail so far. Our present study describes the discovery of a second acidic mammalian chitinase named AMCase. This enzyme is also able to degrade artificial chitin-like substrates as well as chitin from crab shell and chitin as present in the fungal cell wall.

Sequence homology, conservation of intron-exon boundaries and chromosomal location suggest that the genes of members of the mammalian chitinase protein family evolved from a common ancestor by duplication. This is also suggested by their structural similarities, in particular between AMCase and human chitotriosidase. Both are members of family 18 of glycosyl hydrolases, showing an 8-stranded $\alpha/\beta$ (TIM) barrel catalytic

**A**

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1 GCTTTCCAGTCTGGTGGTGAATCCTCCATAGTCTGAAGCCTTTGTGATAACCACAGAATCAGAACATATAAAAAGCTCTGCGGGACTGGTGCTGACTGCA 100

101 ACC ATG ACA AAG CTT ATT CTC CTC ACA GGT CTT GTC CTT ATA CTG AAT TTG CAG CTC GGC TCT GCC TAC CAG CTG 175
  1   M  T  K  L  I  L  L  T  G  L  V  L  I  L  N  L  Q  L  G  S  A   Y  Q  L  24

176 ACA TGC TAC TTC ACC AAC TGG GCC CAG TAC CGG CCA GGC CTG GGG CGC TTC ATG CCT GAC AAC ATC GAC CCC TGC 250
 25 T  C  Y  F  T  N  W  A  Q  Y  R  P  G  L  G  R  F  M  P  D  N  I  D  P  C  49

251 CTC TGT ACC CAC CTG ATC TAC GCC TTT GCT GGG AGG CAG AAC AAC GAG ATC ACC ACC ATC GAA TGG AAC GAT GTG 325
 50 L  C  T  H  L  I  Y  A  F  A  G  R  Q  N  N  E  I  T  T  I  E  W  N  D  V  74

326 ACT CTC TAC CAA GCT TTC AAT GGC CTG AAA AAT AAG AAC AGC CAG CTG AAA ACT CTC CTG GCC ATT GGA GGC TGG 400
 75 T  L  Y  Q  A  F  N  G  L  K  N  K  N  S  Q  L  K  T  L  L  A  I  G  G  W  99

401 AAC TTC GGG ACT GCC CCT TTC ACT GCC ATG GTT TCT ACT CCT GAG AAC CGC CAG ACT TTC ATC ACC TCA GTC ATC 475
100 N  F  G  T  A  P  F  T  A  M  V  S  T  P  E  N  R  Q  T  F  I  T  S  V  I  124

476 AAA TTC CTG CGC CAG TAT GAG TTT GAC GGG CTG GAC TTT GAC TGG GAG TAC CCT GGC TCT CGT GGG AGC CCT CCT 550
125 K  F  L  R  Q  Y  E  F  D  G  L  D  F  D  W  E  Y  P  G  S  R  G  S  P  P  149

551 CAG GAC AAG CAT CTC TTC ACT GTC CTG GTG CAG GAA ATG CGT GAA GCT TTT GAG CAG GAG GCC AAG CAG ATC AAC 625
150 Q  D  K  H  L  F  T  V  L  V  Q  E  M  R  E  A  F  E  Q  E  A  K  Q  I  N  174

626 AAG CCC AGG CTG ATG GTC ACT GCT GCA GTA GCT GCT GGC ATC TCC AAT ATC CAG TCT GGC TAT GAG ATC CCC CAA 700
175 K  P  R  L  M  V  T  A  A  V  A  A  G  I  S  N  I  Q  S  G  Y  E  I  P  Q  199

701 CTG TCA CAG TAC CTG GAC TAC ATC CAT GTC ATG ACC TAC GAC CTC CAT GGC TCC TGG GAG GGC TAC ACT GGA GAG 775
200 L  S  Q  Y  L  D  Y  I  H  V  M  T  Y  D  L  H  G  S  W  E  G  Y  T  G  E  224

776 AAC AGC CCC CTC TAC AAA TAC CCG ACT GAC ACC GGC AGC AAC GCC TAC CTC AAT GTG GAT TAT GTC ATG AAC TAC 850
225 N  S  P  L  Y  K  Y  P  T  D  T  G  S  N  A  Y  L  N  V  D  Y  V  M  N  Y  249

851 TGG AAG GAC AAT GGA GCA CCA GCT GAG AAG CTC ATC GTT GGA TTC CCT ACC TAT GGA CAC AAC TTC ATC CTG AGC 925
250 W  K  D  N  G  A  P  A  E  K  L  I  V  G  F  P  T  Y  G  H  N  F  I  L  S  274

926 AAC CCC TCC AAC ACT GGA ATT GGT GCC CCC ACC TCT GGT GCT GGT CCT GCT GGG CCC TAT GCC AAG GAG TCT GGG 1000
275 N  P  S  N  T  G  I  G  A  P  T  S  G  A  G  P  A  G  P  Y  A  K  E  S  G  299

1001 ATC TGG GCT TAC TAC GAG ATC TGT ACC TTC CTG AAA AAT GGA GCC ACT CAG GGA TGG GAT GCC CCT CAG GAA GTG 1075
300 I  W  A  Y  Y  E  I  C  T  F  L  K  N  G  A  T  Q  G  W  D  A  P  Q  E  V  324

1076 CCT TAT GCC TAT CAG GGC AAT GTG TGG GTT GGC TAT GAC AAC ATC AAG AGC TTC GAT ATT AAG GCT CAA TGG CTT 1150
325 P  Y  A  Y  Q  G  N  V  W  V  G  Y  D  N  I  K  S  F  D  I  K  A  Q  W  L  349

1151 AAG CAC AAC AAA TTT GGA GGC GCC ATG GTC TGG GCC ATT GAT CTG GAT GAC TTC ACT GGC ACT TTC TGC AAC CAG 1225
350 K  H  N  K  F  G  G  A  M  V  W  A  I  D  L  D  D  F  T  G  T  F  C  N  Q  374

1226 GGC AAG TTT CCC CTA ATC TCC ACC CTG AAG AAG GCC CTC GGC CTG CAG AGT GCA AGT TGC ACG GCT CCA GCT CAG 1300
375 G  K  F  P  L  I  S  T  L  K  K  A  L  G  L  Q  S  A  S  C  T  A  P  A  Q  399

1301 CCC ATT GAG CCA ATA ACT GCT GCT CCC AGT GGC AGC GGG AAC GGG AGC GGG AGT AGC AGC TCT GGA GGC AGC TCG 1375
400 P  I  E  P  I  T  A  A  P  S  G  S  G  N  G  S  G  S  S  S  S  G  G  S  S  424

1376 GGA GGC AGT GGA TTC TGT GCT GTC AGA GCC AAC GGC CTC TAC CCC GTG GCA AAT AAC AGA AAT GCC TTC TGG CAC 1450
425 G  G  S  G  F  C  A  V  R  A  N  G  L  Y  P  V  A  N  N  R  N  A  F  W  H  449

1451 TGC GTG AAT GGA GTC ACG TAC CAG CAG AAC TGC CAG GCC GGG CTT GTC TTC GAC ACC AGC TGT GAT TGC TGC AAC 1525
450 C  V  N  G  V  T  Y  Q  Q  N  C  Q  A  G  L  V  F  D  T  S  C  D  C  C  N  474

1526 TGG GCA TAAACCTGACCTGGTCTATATTCCCTAGAGTTCCAGTCTCTTTGCTTAGGACATGTTGCCCCTACCTAAAGTCCTGGAATAAAATCAGCA 1622
475 W  A  *

1623 GTC                                                                                                  1625
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**B**

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h-AMCase          1   YQLTCYFTNWAQYRPGLGRFMPDNIDPCLCTHLIYAFAGRQNNE  44
m-AMCase          1   YNLICYFTNWAQYRPGLGSFKPDDINPCLCTHLIYAFAGMQNNE  44
h-Chitotriosidase 1   AKLVCYFTNWAQYRQGEARFLPKDLDPSLCTHLIYAFAGMTNHQ  44

                  45  ITTIEWNDVTLYQAFNGLKNKNSQLKTLLAIGGWNFGTAPFTAM  88
                  45  ITTIEWNDVTLYKAFNDLKNRNSKLKTLLAIGGWNFGTAPFTTM  88
                  45  LSTTEWNDETLYQEFNGLKKMNPKLKTLLAIGGWNFGTQKFTDM  88

                  89  VSTPENRQTFITSVIKFLRQYEFDGLDFDWEYPGSRGSPPQDKH  132
                  89  VSTSQNRQTFITSVIKFLRQYGFDGLDLDWEYPGSRGSPPQDKH  132
                  89  VATANNRQTFVNSAIRFLRKYSFDGLDLDWEYPGSQGSPAVDKE  132

                 133  LFTVLVQEMREAFEQEAKQINKPRLMVTAAVAAGISNIQSGYEI  176
                 133  LFTVLVKEMREAFEQEAIESNRPRLMVTAAVAGGISNIQAGYEI  176
                 133  RFTTLVQDLANAFQQEAQTSGKERLLLSAAVPAGQTYVDAGYEV  176

                 177  PQLSQYLDYIHVMTYDLHGSWEGYTGENSPLYKYPTDTGSNAYL  220
                 177  PELSKYLDFIHVMTYDLHGSWEGYTGENSPLYKYPTETGSNAYL  220
                 177  DKIAQNLDFVNLMAYDFHGSWEKVTGHNSPLYKRQEESGAAASL  220

                 221  NVDYVMNYWKDNGAPAEKLIVGFPTYGHNFILSNPSNTGIGAPT  264
                 221  NVDYVMNYWKNNGAPAEKLIVGFPEYGHTFILRNPSDNGIGAPT  264
                 221  NVDAAVQQWLQKGTPASKLILGMPTYGRSFTLASSSDTRVGAPA  264

                 265  SGAGPAGPYAKESGIWAYYEICTFLKNGATQGWDAPQEVPYAYQ  308
                 265  SGDGPAGAYTRQAGFWAYYEICTFLRSGATEVWDASQEVPYAYK  308
                 265  TGSGTPGPFTKEGGMLAYYEVCSW--KGATKQRIQDQKVPYIFR  306

                 309  GNVWVGYDNIKSFDIKAQWLKHNKFGGAMVWAIDLDDFTGTFCN  352
                 309  ANEWLGYDNIKSFSVKAQWLKQNNFGGAMIWAIDLDDFTGSFCD  352
                 307  DNQWVGFDDVESFKTKVSYLKQKGLGGAMVWALDLDDFAGFSCN  350

                 353  QGKFPLISTLKKALGLQSASCTAPAQPIEPITAAPSGSGNGSGS  396
                 353  QGKFPLTSTLNKALGISTEGCTAPDVPSEPVTTPP----GSGSG  393
                 351  QGRYPLIQTLRQELSLPYLPSGTPELEV-PKPGQPSEP------  387

                 397  SSSGGSSGGSGFCAVRANGLYPVANNRNAFWHCVNGVTYQQNCQ  440
                 394  GSSGGSSGGSGFCADKADGLYPVADDRNAFWQCINGITYQQHCQ  437
                 388  -EHGPSPGQDTFCQGKADGLYPNPRERSSFYSCAAGRLFQQSCP  430

                 441  AGLVFDTSCDCCNWA                              455
                 438  AGLVFDTSCNCCNWP                              452
                 431  TGLVFSNSCKCCTWN                              445
```

core structure (37, 38). Like chitotriosidase, AMCase contains a N-terminal catalytic core domain of 39 kDa and a C-terminal chitin binding domain separated by a hinge region (11). An ongoing crystallographic study on the three-dimensional structures of human chitotriosidase and AMCase (collaboration with F. Fusetti and B. Dijkstra from the University of Groningen, The Netherlands) should answer some intriguing questions. For example, the molecular basis for the profound differences in stability and catalytic capacity at low pH between the enzymes has to be resolved. It will also be of interest to establish whether the difference in migration of the two enzymes upon SDS-PAGE at nonreducing conditions is caused by differences in disulfide bonds. All 10 cysteines residues in chitotriosidase are conserved in mouse AMCase. The primary amino acid sequence of mouse AMCase shows the presence of 2 additional cysteines in the catalytic core, which are conserved in the human AMCase. Tjoelker *et al.* (11) have recently shown that all 6 cysteines in the chitin-binding domain of human chitotriosidase are involved in disulfide bonds within this domain and are essential for lectin activity.

In view of our observation that mouse and human AMCase mRNA is highly expressed in the stomach, the noted acidic pH optimum and profound acid stability of AMCase is not surprising. The extreme environment in these parts of the gastrointestinal tract requires such special features. The fact that no AMCase mRNA was detected in the intestine suggests that the protein present in these lower parts of the gastrointestinal tract may originate from the stomach and submaxillary glands. However, AMCase EST clones have been identified in the mouse caecum, tongue, and pancreas recently, indicating that several additional parts of the gastrointestinal tract are involved in the generation of AMCase. Whether the observed chitinase activity in the saliva of patients with periodontal inflammation described by van Steijn *et al.* (39) can be ascribed to AMCase remains to be established.

We also observed that AMCase mRNA is expressed in the lung (although to a lesser extent than in the stomach) and that enzyme activity is detectable there. At present the exact cellular sources of AMCase are unknown. Recently Guoping *et al.* (40) identified a silica-induced bronchoalveolar lavage protein with fibroblast growth promoting activity in the rat. This protein is identical to the AMCase we isolated from the rat.[3] It has been shown that the protein could be identified in alveolar macrophages of silicotic rats (40), suggesting that at least in the rat lung this enzyme could be generated by macrophages. However, we have been unable to demonstrate any chitinolytic activity in rat alveolar macrophages (not shown). This could indicate that alveolar macrophages are only capable of producing AMCase under a specific stimulus. Moreover, we have also not observed any expression of AMCase in human monocyte-derived macrophages, even under conditions when the cells massively produce chitotriosidase. A detailed characterization of the promoter regions of AMCase and chitotriosidase is required to understand the selective expression of these enzymes. *In situ* hybridization analysis has to reveal which cells in the respiratory and gastrointestinal tract can express AMCase.

For several vertebrates and invertebrates the presence of chitinase activity in the gastrointestinal tract has been reported (for an overview see Refs. 6, 41, and 42). This activity has sometimes been ascribed to the microorganisms present in the tract. However, gut chitinases have been cloned from several insect species and are thought to be involved in maintenance of the peritrophic matrix (43–45). The peritrophic matrix is a chitinous extracellular layer that surrounds a food bolus in the guts of most arthropods (46), providing a physical barrier to pathogens, facilitating digestion, and protecting against damage by food particles. Our study shows that, at least in rodents and man, a part of the chitinolytic activity found in the gut should be ascribed to an endogenous source also.

The presence of chitinase activity in vertebrates has actually been described earlier, but little is known about the corresponding proteins (6, 42, 47). Place (48) described the purification of a rainbow trout chitinase, which was isolated from the cardiac portion of the stomach. Comparison of the first 26 amino acids of this fish chitinase showed that it is 54% identical to mouse AMCase. Comparison of the complete sequence should reveal more information regarding the evolutionary relationship between the mammalian and fish stomach chitinases.

At present the physiological function of AMCase is unknown. Our study has revealed a remarkable parallel between chitinases and another group of endo-glucosaminidases, the lysozymes. It is well known that distinct lysozyme isoforms occur in various organisms. Lysozymes produced by phagocytes are basic proteins that fulfill a defense function by virtue of their ability to degrade the cell wall of Gram-negative bacteria. In the gastrointestinal tract of some species, acidic lysozymes are expressed that are acid stable and active at low pH. These enzymes are thought to function as food processors (49). By their action, the cell walls of bacteria that ferment plant materials are degraded, allowing the subsequent release and assimilation of their contents. It is conceivable that AMCase also plays a role in food assimilation as earlier proposed for fish chitinases by Lindsay (50), whereas the phagocyte-specific chitotriosidase is primarily involved in defense. The observation that AMCase is also expressed in the lung may point to a dual function for the enzyme, both in defense and food processing.

In ruminant artiodactyls, leaf-eating monkeys, and the bird hoatzin, lysozyme has been adapted by rapid convergent evolution to allow survival and functioning in the acidic, proteolytic environment of the stomach (51). These adaptations changed the global properties of the enzyme by a reduction of the isoelectric point so that the protein is neutral or acidic rather than basic and by a reduction in the number of acid labile bonds and side chains (51). Similar differences can be observed between chitotriosidase and AMCase, suggesting that the same kind of evolutionary processes played a role in chitinase adaptation.

Because AMCase is a functional chitinase, it is conceivable that the existence of AMCase in man has allowed the high panethnic incidence of deficiency in chitotriosidase. It will be of great interest to study also in detail the precise composition of chitinases and their respective functions in lower vertebrates such as fish.

Our demonstration of a novel chitinolytic member of the mammalian chitinase family that might play an important role in defense and/or nutrition warrants further investigation. Re-

[3] R. G. Boot, E. F. C. Blommaart, E. Swart, K. Ghauharali-van der Vlugt, N. Bijl, C. Moe, A. Place, and J. M. F. G. Aerts, unpublished observation.

Fig. 8. **Human AMCase cDNA sequence and deduced amino acid sequence.** *A*, the human AMCase cDNA sequence (GenBank™ accession number AF290004) is indicated by the *upper sequence*, and the deduced amino acid sequence is indicated *below* the nucleotide sequence. The characteristic hydrophobic signal peptide (amino acids 1–21) is *underlined* with a *single line*. *B*, amino acid sequence comparison of mature (without signal peptide) human (*h*) and mouse (*m*) AMCase and human chitotriosidase. Residues conserved among at least two out of the three sequences are *boxed*.

search on structural properties, regulation of expression, and the evolutionary relationship of the different members of the mammalian chitinase family could give insights into the physiological role of these interesting proteins.

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# Identification of a Novel Acidic Mammalian Chitinase Distinct from Chitotriosidase

Rolf G. Boot, Edward F. C. Blommaart, Erwin Swart, Karen Ghauharali-van der Vlugt, Nora Bijl, Cassandra Moe, Allen Place and Johannes M. F. G. Aerts

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