Chitinases of the Avian Malaria Parasite Plasmodium gallinaceum, a Class of Enzymes Necessary for Parasite Invasion of the Mosquito Midgut*

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The Plasmodium ookinete produces chitinolytic activity that allows the parasite to penetrate the chitin-containing peritrophic matrix surrounding the blood meal in the mosquito midgut. Since the peritrophic matrix is a physical barrier that the parasite must cross to invade the mosquito, and the presence of allosamidin, a chitinase inhibitor, in a blood meal prevents the parasite from invading the midgut epithelium, chitinases (3.2.1.14) are potential targets of malaria parasite transmission-blocking interventions. We have purified a chitinase of the avian malaria parasite Plasmodium gallinaceum and cloned the gene, PgCHT1, encoding it. PgCHT1 encodes catalytic and substrate-binding sites characteristic of family 18 glycohydrolases. Expressed in Escherichia coli strain AD494 (DE3), recombinant PgCHT1 was found to hydrolyze polymeric chitin, native chitin oligosaccharides, and 4-methylumbellifere derivatives of chitin oligosaccharides. Allosamidin inhibited recombinant PgCHT1 with an IC50 of 7 μM and differentially inhibited two chromatographically separable P. gallinaceum ookinete-produced chitinase activities with IC50 values of 7 and 12 μM, respectively. These two chitinase activities also had different pH activity profiles. These data suggest that the P. gallinaceum ookinete uses products of more than one chitinase gene to initiate mosquito midgut invasion.

Defining molecular targets for drug or vaccine intervention remains a key strategy for developing new ways to prevent and treat malaria, a disease that exacts an enormous social and economic toll worldwide. One such target is the Plasmodium spp. chitinase (EC 3.2.1.14). Chitinases are found in prokaryotes and eukaryotes (1); their biologic roles include cell wall modification (e.g. fungi (2), Entamoeba (3) and filaria parasites (4), carbon source degradation (e.g Streptomyces spp. (5, 6), Serratia marcescens (7), and Vibrio spp. (8)) and plant and fungal host defense against chitin-containing pathogens (1). One other protozoan pathogen of man, Leishmania donovani, the agent of human visceral leishmaniasis, is known to use a chitinase in its life cycle (9, 10). The Leishmania chitinase is thought to disrupt the sand fly cardiac valve, allowing amastigotes to be regurgitated from the midgut into the skin of the vertebrate host. The Leishmania chitinase is not thought to function in invasion of the arthropod vector per se (11). In contrast, Plasmodium chitinase is thought to be required for the parasite to invade the mosquito midgut after being taken up in a blood meal (12). Because of its critical biological function in the life cycle of the malaria parasite, the Plasmodium chitinase is a potential target for blocking transmission from the vertebrate host to the mosquito vector (12).

How the Plasmodium ookinete penetrates the chitin-containing peritrophic matrix to begin its invasion of the mosquito has been outlined. By using transmission electron microscopy, Sieber et al. (13) demonstrated that the motile ookinete, fully mature 20–25 h after zygote formation, actively penetrates the peritrophic matrix (PM)1 in the mosquito midgut en route to invading the midgut epithelium. Huber et al. (14) showed that the Plasmodium gallinaceum ookinete secretes chitinolytic activity. Native activity gel electrophoresis of ookinete cell extracts and culture supernatants showed two predominant bands of chitinase activity, but the precise molecular masses of the two forms could not be determined because active enzyme could not be obtained after SDS-PAGE. Shahabuddin et al. (12) showed that addition of an oligosaccharide chitinase antagonist, allosamidin, to a blood meal prevents oocyst formation in the mosquito, in both the Aedes aegypti-P. gallinaceum and the Anopheles freeborni Plasmodium falciparum model systems. This effect could be completely reversed by enzymatic degradation of the PM in vivo, by adding exogenous chitinase to the blood meal. These observations demonstrated that a chitinase is necessary for malaria parasites to invade the mosquito and

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1 The abbreviations used are: PM, peritrophic matrix; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; 4-MU, 4-(2-aminoethyl)benzenesulfonyl fluoride; PCR, polymerase chain reaction; bp, base pair; Ni-NTA, nickel-nitrilotriacetic acid; Endo, endoproteinase.
P. gallinaceum can be obtained as a useful model system for studying malaria parasite transmission (19, 20).

Because of intrinsic biologic interest and the potential for Plasmodium chitinases to be targets of interfering with malaria transmission (for a review of malaria transmission-blocking vaccines and the potential of Plasmodium chitinases as targets, see Refs. 15 and 16), we sought to characterize the malaria parasite chitinase genes. We began our studies with P. falciparum (17, 18). Conveniently, large numbers of P. gallinaceum chitinase-producing ookinotes can be obtained in vitro, allowing for the direct study of the protein. In addition, P. gallinaceum serves as a useful model system for studying malaria parasite transmission (19, 20).

**EXPERIMENTAL PROCEDURES**

**Preparation of P. gallinaceum Ookinite Chitinase**—The 8A strain of P. gallinaceum was used to infect 4–6-week-old White Leghorn chickens. A gametocyte-producing line was maintained by subpassage in chickens and periodic passage through mosquitoes. Ookinotes were cultured from purified zygotes in serum-free and protease-free M199 culture medium as described previously (21). Preparations routinely yielded 5–15 × 10⁷ ookinetes per 5 chickens, with transformation efficiencies of 50–90%.

Twenty four to 30 h cultures of ookinetes were centrifuged, and the pellet and supernatants were pooled separately and frozen at −20 °C. Extracts of ookinetes were prepared by addition of 20 mm sodium phosphate, pH 6.8, to the ookinite cell pellet, usually without protease inhibitors, followed by vigorous vortexing, three cycles of freeze-thawing (dry ice to room temperature), and sonication (6 cycles for 20 s on ice). For the experiment in which the time course of chitinase expression was determined with Western immunoblotting, a mixture of protease inhibitors was added directly to the fresh cell pellet in 20 mm sodium phosphate, pH 6.8 (2 mM AEBSF, 5 mm EDTA, 200 μM N-tosyl-1,2-diisopropyl carbonylfluoromethyl ketone, 100 μM tosyl-1-tosylamido-3-methane ethyl ketone, 1 mg/ml pepstatin, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 1% Triton X-100). Pooled supernatants were 200-fold concentrated by centrifugal ultrafiltration (Centriprep 10, Amicon, Beverly, MA), diafiltered against 1% Triton X-100, 1% Triton X-100 (Microfluor B, Catalog #011-010-7205, Dynatek, Chantilly, VA), 10 mm 4-methylumbelliferyl-α-D-glucopyranoside, and tryptic digestion (Promega) (22), and a 10% aliquot of the resultant mixture was analyzed. Sequence information was determined with capillary (180 μm × 15 cm column, LC Packings, Amsterdam) reverse-phase chromatography coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnmixon LCQ, San Jose, CA). The instrument was programmed to acquire successive sets of three scan modes consisting of full scan MS over the m/z 395–1200 atomic mass unit, followed by two data-dependent scans on the most abundant ion in that full scan. These data-dependent scans allowed the automatic acquisition of a high resolution (zoom) scan to determine charge state and existence of co and MS/MS spectra for peptide sequence information. The remainder (90%) of the peptide mixture was separated by microbore high performance liquid chromatography using a Zorbax C18 1.0 × 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions were chosen based on differential UV absorbance at 205, 277, and 292 nm, peak symmetry, and resolution and then further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Thermo BioAnalysis Lasermat 2000 (Hemel, UK). Strategies for peak selection, reverse-phase separation, and Edman microsequencing have been previously described (22). Tryptic peptides were submitted to automated Edman degradation of a Perkin-Elmer/ Applied Biosystems 477A or Procise 494-HIT protein sequencer (Foster City, CA). When necessary, complementary Edman degradation data and MS/MS spectra were used to improve the final sequence interpretation.

**Determination of DNA Sequence of Chitinase Gene and Non-translated Flanking Sequence**—Degrenerate oligodeoxynucleotides were designed based on the amino acid sequences of the following peptides (Fig. 2): GST29 (His to Lys) (CA/CT) TA(TC) TA(TC) TA(TC) AA(TC) AA(CT) AC(GA/CT) TA(TC) AAAA), GST33 (Asn to Lys) (AA(G/A) CC(A/G) GT(A/C) AA(A/G) AC(A/G) C) (I represents inosine). Total RNA was isolated from 24-h post-exflagellation ookinetes with Trizol (Life Technologies, Inc.). First-strand cDNA synthesis, using 1 μg total RNA, was prepared using the Capfinder system (CLONTECH, Palo Alto, CA). For the experiment with a Perkin-Elmer 9800 thermal cycler and KlenTaq DNA polymerase (CLONTECH), the following polymerase chain reaction (PCR) cycling conditions were used: 94 °C for 3 min for 1 cycle; 94 °C for 30 s, 47 °C for 30 s, 68 °C for 3 min for 35 cycles; 4 °C on hold.

The full-length transcript was PCR-amplified using the ookinete first-strand cDNA prepared as described above as template (Fig. 2). To amplify the 5′ end of the transcript, an antisense nondegenerate gene-specific oligonucleotide primer 2501 was synthesized (GGG TTT TCA of acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Amino-terminal Peptide Sequence Analysis—For purified, native, P. gallinaceum ookinite-produced chitinase, 10% of the volume of the acetonitrile fractions from the reverse-phase step was used to obtain amino-terminal sequence. Recovery of the purified protein from the HPLC was maximized by adding neat trifluoroacetic acid to a final concentration of 10%. One hundred percent of the recovered protein was applied to the biphasic column of a Hewlett-Packard G1005A (Palo Alto, CA), followed by a 1 ml wash with the manufacturer's sample loading solution. The purified protein was then subjected to automated Edman degradation using the manufacturer's recommended protocol. For amino-terminal sequencing of endoproteinase Lys-C-treated rPgCHT1-NT1, the cleaved recombinant protein was run on SDS-PAGE, electroblotted to PVDF, stained with 0.05% Coomassie Blue in 40% methanol, 1% acetic acid, and destained with 50% methanol. The stained band was excised and submitted to automated Edman degradation on an Applied Biosystems 494/HT ProSe Q Sequence System in the University of Texas Medical Branch Protein Chemistry Core Facility.

**Tryptic Digestion, HPLC Separation, and Microsequencing**—To obtain amino acid sequence of internal tryptic peptide fragments, the remaining 90% of the acetonitrile fractions from the reverse-phase step was electrophotographically purified by SDS-PAGE. 50 μl of 2 × SDS-PAGE sample buffer (Novex, San Diego, CA) was added to each 0.5 ml fraction from the reverse-phase step and reconstituted by vacuum centrifugation (Hetoac, Heto Labs, Denmark). These fractions were size-fractionated by SDS-PAGE on a 4–20% gel (Novex), which was stained with Coomassie Blue R-250 (Bio-Rad) and destained for 4 h with Gel-Stain Destain Solution (Novex) with three changes of destaining solution.

Stained bands were excised from the gel, rinsed twice with 50% acetonitrile in HPLC-grade water, and frozen on dry ice. Subsequent protein sequencing steps were performed at the Harvard University Microchemistry Laboratory (William Lane, Director, Cambridge, MA). The band was subjected to in-gel reduction, S-carboxy-amidomethylation, and tryptic digestion (Promega) (22), and a 10% aliquot of the resultant mixture was analyzed. Sequence information was determined by capillary (180 μm × 15 cm column, LC Packings, Amsterdam) reverse-phase chromatography coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnmixon LCQ, San Jose, CA). The instrument was programmed to acquire successive sets of three scan modes consisting of full scan MS over the m/z 395–1200 atomic mass unit, followed by two data-dependent scans on the most abundant ion in that full scan. These data-dependent scans allowed the automatic acquisition of a high resolution (zoom) scan to determine charge state and existence of co and MS/MS spectra for peptide sequence information. The remainder (90%) of the peptide mixture was separated by microbore high performance liquid chromatography using a Zorbax C18 1.0 × 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions were chosen based on differential UV absorbance at 205, 277, and 292 nm, peak symmetry, and resolution and then further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Thermo BioAnalysis Lasermat 2000 (Hemel, UK). Strategies for peak selection, reverse-phase separation, and Edman microsequencing have been previously described (22). Tryptic peptides were submitted to automated Edman degradation of a Perkin-Elmer/Applied Biosystems 477A or Procise 494-HIT protein sequencer (Foster City, CA). When necessary, complementary Edman degradation data and MS/MS spectra were used to improve the final sequence interpretation.
GTT GTA GTA AGG TC) based on the internal sequence of the PCR product generated by degenerate oligonucleotides derived from the amino acid sequences of GTs4 and GT29; the 5’ PCR primer from the Capfitter kit (AAG CAG TGG TAA CAA CGG AGA GT) was used as the sense primer. Similarly, a sense nondegenerate gene-specific primer 2505 TAA AAA TTT GGA TGG GGC TGC GAA was used to generate an antisense cDNA synthesis primer (T20 A/C/G/A/C/G/T). The reaction protocol was as follows: 95 °C for 3 min for 1 cycle; then 95 °C for 30 s, 54 °C for 30 s, and 68 °C for 3 min for 30 cycles; then 4°C. PCR products were ethanol-purifed and resuspended in water. The DNA was phosphorylated and the ends made blunt (10 μl of 10× React 1 buffer (Life Technologies, Inc.), 10 μl of 10 μM ATP (Promega, Madison, WI), 10 μl of T4 DNA polymerase, 10 units of T4 kinase, water to 100 μl, incubated at 37 °C for 60 min). The products of this reaction were electrophoresed and then purified from a Tris acetate/EDTA agarose gel using the Geneclean II kit (Bio 101, San Diego, CA). The DNA was ligated into μC18 (Smal-digested, bacterial alkaline phosphatase-treated, Amersham Pharmacia Biotech), electro-porated (1.8 kV, 25 microfarads capacitance, 200 ohms resistance) (Gene Pulser, Bio-Rad) into electrocompetent DH10B E. coli (Life Technologies, Inc.), incubated in SOC (Life Technologies, Inc.) at 37 °C in a shaking incubator for 1 h, and plated on LB/ampicillin (100 μg/ml) plates. Plasmids from transformants were isolated by alkaline lysis (Wizard Miniprep, Promega, Madison, WI). Clones containing the appropriate large insert were confirmed by restriction enzyme analysis of the conjugates, sequenced using dye terminator reactions according to the manufacturer’s instructions (DNA Sequencing Kit, Part Number 402079, Perkin-Elmer Applied Biosystems, Foster City, CA) and analyzed by an automated ABI sequencer (ABI Prism, 377 DNA Sequencer, Perkin-Elmer). Independent, cloned full-length genomic DNA and cDNA PCR products were sequenced in their entirety to verify the final sequence.

**Southern Blot Analysis—** *P. gallinaceum* Southern Blot Analysis—P. gallinaceum gDNA (10 μg) was digested with restriction enzymes DdeI, NcoI, EcoRI, and HindIII (Life Technologies, Inc.) in pairs as indicated in Fig. 3 and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). A 42-mer oligonucleotide probe (5 pmol) based on the coding sequence of the open reading frame of the *P. gallinaceum* chitinase (numbered amino acids refer to Fig. 2): 219-5LDGWDIDWEPHGK-232 (active site) and 506-5CGKAAHYNTDYKYE-520 (carboxyl terminus). The probes were chosen based on predicted antigenicity from a Kyte-Doolittle hydrophilicity plot. Peptide synthesis, carrier coupling, and structural analysis was performed by Mark Greenfield and Jan Luszko, NIAID (Twinbrook Facility, Rockville, MD). Peptide purity was verified by reverse-phase HPLC and mass spectroscopy. The peptides were keyhole limpet hemocyanin via the terminal cysteine (carboxyl-terminal peptide) or lysine (active site peptide). Molar coupling ratios of peptidedecarrier, as determined by amino acid analysis, were 39 for the carboxyl-terminal peptide and 413 for the active site peptide.

Animal-use protocols for obtaining polyclonal mouse antisera were approved by the Animal Care and Use Committee, NIAID, National Institutes of Health. Mice were immunized according to the following schedule: each mouse received an intraperitoneal injection of 100 μg of conjugate emulsified in 100 μl of complete Freund’s adjuvant (Sigma) for primary immunization, followed by 50 μg of conjugate emulsified in 100 μl of incomplete Freund’s adjuvant three times at 3-week intervals.

For immunoblotting, polypeptides were separated on 4–20% SDS-PAGE gels (Novex) and electroblotted to nitrocellulose using the Novex Xcell Blot II module. After blocking with 5% dried skim milk in PBS, 0.05% Tween 20 (PBS-T), blots were incubated in primary polyclonal antisera at 1:1000 dilution in PBS, 0.05% Tween 20 (PBS-T), blots were incubated in primary polyclonal antisera at 1:1000 dilution in PBS, 0.05% Tween 20 for 1 h at room temperature. Membranes were washed over 3 times with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h. After three washes over 20 min with PBS-T, blots were incubated in secondary antibody (goat anti-mouse IgG heavy and light chain, alkaline phosphatase-conjugated, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:5000 dilution in PBS, 0.05% Tween 20, for 1 h.

**Analysis of Chitinase Activity—** Chitinase activity of both native and recombinant chitinases was measured in the supernatant after each enzyme reaction. The enzyme preparations were analyzed for their ability to degrade polymeric chitin, as described previously (14). Second, microfluorimetry (HTS7000, Perkin-Elmer, excitation 360 nm and emission 465 nm) was used to measure the hydrolysis of 4-MU GlcNAc, 4-MU GlcNAc2, 4-MU GlcNAc3, and 4-MU GlcNAc4 (Sigma) as described previously. Initial enzyme reaction rates were measured. Enzymatic activity is reported as relative fluorescence units or fold change. Third, TLC was used to analyze the products of recombinant or okiniete-produced *P. gallinaceum* chitinase using native chitin oligosaccharides and synthetic 4-MU derivatives of chitib oligosaccharides as substrates. With native chitin oligosaccharides (GlcNAc<sub>6</sub>-Calbiochem), 6 μl of 5 mM substrate was mixed with 4 μl of 5× citrate/phosphate, pH 3–7 (McIlvaine buffer), to which was added 10 μl of enzyme. The reaction mixtures were incubated at 37 °C overnight and then analyzed by TLC. 3 μl of the reaction mixture were applied to Silica Gel-60 TLC plates, 20 × 20 cm (EM Science, Gibbstown, NJ) and chromatographed in isopropl alcohol:ethanol:water (5:2:1). The plates were developed by spraying the plates with 10% sulfuric acid in ethanol followed by heating at 120 °C for 10 min to detect dark spots. The chromatograms were scanned on a flat-bed scanner and images analyzed using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA). Samples of enzyme reacted with synthetic 4-MU substrates (5 μl, containing 0.5 nmol of substrate/product) were applied to 10-cm Silica Gel-60 TLC plates following overnight incubation at 37 °C. Products were separated as above. These chromatograms were visualized with a FLUOR-5 imager using 366 nm excitation/456 nm emission filters (Bio-Rad).

**Protease Treatment of Recombinant NT1-PgCHT1—** In triplicate, aliquots of rPgCHT1 were incubated with sequence specific endoproteinase Lys-C (Promega, Madison, WI) or with 1 μl of 10× Proteinase K solution at 37 °C for 2 h (Promega, Madison, WI). The reactions were stopped with 250 μl imidazole in a buffer of 20 μs Tris, pH 8.0, 300 μM NaCl, after first washing with 10 column volumes of 10 μM and then 20 μM imidazole in the same buffer.
Identification of a Fragment of a P. falciparum Chitinase—824 bp of preliminary sequence data of the P. falciparum chitinase, found by random shotgun sequencing in the chromosome 14 sequencing project, was obtained from The Institute for Genomic Research website. The plasmid clone, PNADI77, was obtained from The Institute for Genomic Research and the entire insert sequenced. The Institute for Genomic Research sequence, plus the additional sequence that we determined, has been submitted to GenBank™ with accession number AF072442. The sequencing of chromosome 14 was part of the International Malaria Genome Sequencing Project.

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RESULTS

Purification of the P. gallinaceum chitinase—A mixture of supernatants and soluble extracts of 2 × 10⁹ P. gallinaceum ookinetes was sequentially subjected to anion exchange, hydrophobic interaction, and reverse-phase HPLC (Fig. 1). 4-MU GlcNAc₃ was used as the substrate for following endochitinase activity through the anion exchange and hydrophobic interaction steps; acetonitrile/trifluoroacetic acid in the reverse-phase step irreversibly disrupted the chitinase activity. Coomassie Blue staining of the

## FIG. 1. Purification of the P. gallinaceum chitinase—a, quaternary ammonium anion exchange HPLC of crude ookinete extracts. The gradient from 20 to 500 mM NaCl was developed over 30 min. Chitinase-containing fractions (*) eluted at 150–250 mM NaCl. b, hydrophobic interaction HPLC. Chitinase-containing fractions from a were injected into a phenyl-TSK hydrophobic interaction column. Chitinase-containing fractions eluted 18 min after the ammonium sulfate concentration reached 0 M. c, because of a high base line, chitinase-containing fractions from b were re-injected onto the same hydrophobic interaction column (HIC). d, chitinase-containing fractions from c were injected into a C18 reverse-phase HPLC column. e, SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining of fractions from d. Solid lines in the panels indicate absorbance; lines with ◆ indicate chitinase activity.

Determinaton of pH Activity Profiles and Allosamidin Inhibition Curves—By using a citrate/sodium phosphate buffer with pH ranging from 3.0 to 7.0 in 0.5 pH unit increments, aliquots of enzyme were incubated with 4-MU GlcNAc₃ (100 μM). Chitinase reaction rates were analyzed by microfluorimetry. Allosamidin (courtesy of Dr. Shosei Sakuda, University of Tokyo, Japan, and Dr. Jon Mynderse, Eli Lilly and Co., Indianapolis) was made as a 2 mg ml⁻¹ stock in water and diluted.

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Computer Analysis—Analysis of DNA sequences was performed using the Lasergene set of programs (DNASTAR, Madison, WI). Homology searches were performed with gapped BLAST, with further profile analysis performed with PSI BLAST. The multiple alignments were generated using the GIBBS sampling procedure. The signal sequence was predicted using the algorithm of von Heijne (25).

Ten percent of the pooled reverse-phase fractions 62–64 was subjected to immunoblot analysis, antisera to the E. histolytica chitinase active site recognized the 60-kDa doublet (26). When an aliquot of pooled fractions 62–66 was subjected to immunoblot analysis, antisera to the E. histolytica chitinase active site recognized the 60-kDa doublet in fractions 62–64 and the 60 and 210-kDa proteins in fractions 65–66 (data not shown).

Direct Edman degradation of an aliquot of pooled fractions 62–64 was analyzed by direct amino-terminal sequencing. The remaining 90% was lyophilized in the presence of SDS, subjected to SDS-PAGE, and stained with Coomassie Blue. The purified doublet was excised from the gel and subjected to microsequencing.

Direct Edman degradation of an aliquot of pooled fractions 62–64 (Fig. 1e; 5 pmol of protein as determined by amino acid analysis) showed two distinguishable amino-terminal sequences (amino-terminal 1 and amino-terminal 2, Fig. 3). Three peptides, GT29, GT33, and GT84 (Fig. 3), were produced by in situ trypsin digestion of the purified protein doublet and isolated by reverse-phase HPLC; these protein fragments were further analyzed by mass spectroscopy and Edman degradation.

Determination of the DNA Sequence of the P. gallinaceum Chitinase Gene, PgCHT1, That Encodes the 60-kDa Chitinase—Degenerate oligodeoxynucleotide primers were synthe-
and gave rise to the full-length 2508-bp cDNA of PgCHT1 (GenBank accession number AF064079). Primer 2501 paired with the Capfinder system as template (Fig. 2). The first PCR reaction used the primer pair 2503 oligo(dT) primer (see “Experimental Procedures”) synthesized based on the tryptic peptide sequences of mature *P. gallinaceum* ookinete total RNA and degenerate oligodeoxynucleotide primers based on the amino acid sequences of GT29 and GT84, respectively, generated single products that were cloned and sequenced (data not shown). These PCR fragments encoded amino acid sequences without recognizable homology on BLAST search. Nondegenerate gene-specific oligodeoxynucleotides, synthesized based on the internal sequences of these PCR products, were used as primers in two separate PCR reactions; these reactions used first-strand cDNA of mature *P. gallinaceum* ookinete, prepared with the CLONTECH Capfinder system as template (Fig. 2). The first PCR reaction used the CLONTECH 5’ Capfinder PCR primer and a gene-specific antisense primer; similarly, the second PCR used the oligo(dT) primer as the 3’ antisense primer and a gene-specific 3’ PCR primer; the resulting PCR product amplified the 3’ end of the cDNA template, a single band was obtained, cloned, and sequenced; when amplifying the 3’ end, several bands were obtained, and only the highest molecular weight band was cloned and sequenced (data not shown). These two sequences (using PCR primers to cross the overlap between the two gene-specific primers) were combined to produce a 2508-bp cDNA corresponding to the full-length mRNA transcript from the initiation of transcription, a Kozak consensus sequence AAA(A/A)TG at the predicted transcriptional start site, or the presumptive site of the initiation of transcription, consistent with a single or low copy number copy gene (data not shown). These two sequences (using PCR primers to cross the overlap between the two gene-specific primers) were combined to produce a 2508-bp cDNA corresponding to the full-length mRNA transcript from the initiation of transcription, a Kozak consensus sequence AAA(A/A)TG at the predicted transcriptional start site typical of *Plasmodium* spp. is present (28). A secretory signal sequence of 22 amino acids is predicted, followed by a 42-amino acid lysine/asparagine-rich precursor region not found in the enzymatically active, purified 60-kDa chitinase doublet. Amino-terminal sequences 1 and 2 are co-linear near the amino terminus. The two forms of the purified chitinase are predicted to be of molecular mass 60,595 and 59,117 Da, respectively, similar to the apparent molecular masses observed for the protein doublet on SDS-PAGE (Fig. 1). BLAST search with the full-length *P. gallinaceum* amino acid sequence revealed homologies to numerous other chitinases; similarities were limited to the substrate binding and catalytic active site motifs (Fig. 4). On searching the *P. falciparum* chromosome 14 genome data base (29), a partial sequence of a *P. falciparum* gene encoding a family 18 glycohydrolase catalytic domain was found. This *P. falciparum* fragment was approximately 1000 bp (Fig. 4) and encoded motifs characteristic of the substrate-binding and catalytic active sites of family 18 glycohydrolases (Fig. 4) (30). This *P. falciparum* chitinase has been further characterized (31). The substrate-binding site (amino acids 187–194) contains the consensus sequence XXXXXG, where X represents hydrophobic amino acids, except at the amino-terminal end where there is an unusual non-conservative isoleucine substitution for a lysine. Both *Plasmodium* chitinases are typical of family 18 glycohydrolases, with one exception: uniquely the *Plasmodium* chitinases lack a highly conserved tyrosine (or a conservative change such as phenylalanine) at position 229. A putative chitin-binding domain is present that, although not sharing significant amino acid sequence similarity with chitin-binding domains of any eukaryotic chitinases, has significant secondary structural homology to bacterial chitin-binding domains (data not shown). Analysis of Different Forms of *P. gallinaceum* Chitinases and Time Course of Expression—To confirm further that the *P. gallinaceum* gene sequence determined above encoded the purified 60-kDa doublet that we identified as a chitinase, antisera for use in Western immunoblots were prepared to synthetic
peptides consisting of the catalytic active site and a peptide near the carboxyl terminus (Fig. 3). Antibody specificity of antisera raised against the synthetic peptides derived from the *P. gallinaceum* 60-kDa chitinase was confirmed by Western immunoblotting. *P. gallinaceum* zygotes and ookinetes with immune and preimmune sera against both synthetic peptides; immune sera recognize proteins in ookinetes but not zygotes, as determined by analysis with Western immunoblot; all of these proteins react with antisera raised to a synthetic peptide derived from the active site of *PgCHT1* (Fig. 5b, left panel). This antisera lacked reactivity to *P. gallinaceum* zygote extracts (Fig. 5b, left panel) and non-recombinant *E. coli* extracts (data not shown). Only a subset of these bands (160-, 66-, and 60-kDa doublet) react with antisera directed against the carboxyl-terminal region (Fig. 5b, right panel); the 66-kDa band (thin arrow) is the precursor to the 60-kDa doublet as shown by Western immunoblot; ookinete extracts with antisera raised to a synthetic peptide from the region between the signal peptide and the NT1 cleavage site (data not shown). The appearance of these bands correlates with the appearance of chitinase activity during the development of zygotes to ookinetes (Fig. 5c), consistent with the hypothesis that these bands are those of chitinases.

*P. gallinaceum* ookinetes produce a number of proteins (210-, 160-, 66-, 60-kDa doublet, 35-kDa) that are stage-specifically expressed in mature ookinetes and are not present in zygotes, as determined by analysis with Western immunoblot; all of these proteins react with antisera raised against the synthetic peptide derived from the active site of *PgCHT1* (Fig. 5b, left panel). This antisera lacked reactivity to *P. gallinaceum* ookinete extracts (Fig. 5b, left panel) and non-recombinant *E. coli* extracts (data not shown). Only a subset of these bands (160-, 66-, and 60-kDa doublet) react with antisera directed against the carboxyl-terminal region (Fig. 5b, right panel); the 66-kDa band (thin arrow) is the precursor to the 60-kDa doublet as shown by Western immunoblot; ookinete extracts with antisera raised to a synthetic peptide from the region between the signal peptide and the NT1 cleavage site (data not shown). The appearance of these bands correlates with the appearance of chitinase activity during the development of zygotes to ookinetes (Fig. 5c), consistent with the hypothesis that these bands are those of chitinases.

### Figure 4

**Sequence alignment of bacterial and eukaryotic chitinases.** Each block of amino acids corresponds to β-barrel structures (β1–β8) predicted on the basis of the three-dimensional structures of the *S. marcescens* chitinase and the plant-defense protein hevamine to be homologous. Conserved amino acids are indicated in color: yellow, hydrophobic side chains; brick red, polar side chains; blue background, small side chains; green background, charged. Numbers between blocks of amino acids indicate gaps. Below the protein sequences are consensus positions identified as follows: s, amino acids with small side chains (A, G, S, I, V, N, Y); a, amino acids with aromatic side chains (F, Y, W); p, amino acids with polar side chains (K, R, H, E, D, Q, N); t, amino acids with charged side chains (E, D, K, H, R); u, amino acids with tiny side chains (G, A, S). Capital letters refer to specific amino acids. GenBank™ accession numbers are indicated next to the species abbreviations, which are as follows: *Pg*, *P. gallinaceum*; *Pf*, *P. falciparum*; *As*, *A. aegypti*; *Ld*, *L. donovani*; *Aha*, *A. haemokunsiis*; *Bli*, *B. licheniformis*; *Ce*, *C. elegans*; *Hs*, *H. sapiens*; *Ec*, *E. coli*; *Bs*, *B. subtilis*; *Cpp*, *C. paraputrificum*; *Cooimi*, *C. thermocellum*; *ChT-NPV*, nuclear polyhedrosis virus; *Aeg*, *A. eggyptii*; *Brasi*, *B. amyloliquefaciens*; *Vih*, *V. harveyi*. The'LPM'-barrel structures of these proteins are graphically and immunologically distinct chitinases—ini-
The separate peaks of chitinase activity were concentrated, subjected to SDS-PAGE under both reducing and non-reducing conditions, and Western immunoblotted using antisera that recognize the catalytic active site and carboxyl terminus (Fig. 6, b–c). Several proteins reacted with antibody to the active site of PgCHT1. Western immunoblot of peak 1 (fractions 35–37) with active site antisera demonstrated the 60-kDa chitinase doublet both under non-reducing (Fig. 6b) and reducing (Fig. 6d) conditions. In addition, a 35-kDa protein in peak 1, of unknown function, reacted with the active site antisera. This 35-kDa protein is stage-specifically expressed in ookinetes, is not found in zygotes (Fig. 5b, left panel), and could be either a degradation product of another chitinase protein, the product of another chitinase gene, or simply an unrelated protein. Western immunoblot of peak 2 (fractions 42–44) with active site antisera demonstrated a predominant band of 210 kDa under non-reducing conditions (Fig. 6b) that, under reducing conditions (Fig. 6d), was not detected and was replaced by a 35-kDa protein. In addition, in fraction 42, a band of 160 kDa reacted with antisera to the active site but not the carboxyl terminus (Fig. 6, b–c). This 160-kDa protein is stage-specifically expressed in ookinetes and is not detectable in zygotes (Fig. 6b, both panels). Additional resolution of this second peak of chitinase activity with additional anion exchange chromatography and enzymatic analysis suggests that both the 210- and the 160-kDa proteins are chitinases (data not shown). The active site antisera recognized both the 210- and 35-kDa bands, but the carboxyl-terminal antisera recognized neither (Fig. 6, b and c). This finding suggests that the carboxyl-terminal epitope is absent in the 210- and 35-kDa bands in the second peak of chitinase activity, as well as the 35-kDa protein in the first peak of chitinase activity.

**Sites of Action of Endoproteinase Lys-C on the 60-kDa P. gallinaceum Chitinase—** *P. gallinaceum* chitinase has been reported to be secreted as an inactive zymogen that mosquito midgut proteases activate to a fully active enzyme (12); this finding has become established in the literature (12, 32–34). The serine protease endoproteinase Lys-C (Endo Lys-C) was reported to increase the *P. gallinaceum* chitinase activity in culture supernatants up to 13-fold (12). To characterize further this phenomenon, we used Western immunoblotting of native, ookinete-secreted chitinase (Fig. 7) and amino-terminal sequencing of Endo Lys-C-cleaved rPgCHT1 to delineate the sites where Endo Lys-C cleaves the 60-kDa *P. gallinaceum* chitinase.

Concentrated ookinete supernatants were treated with Endo Lys-C and the reactions terminated with the serine protease inhibitor, AEBSF. We found that chitinase activity, as assessed by 4-MU GlcNAc₃ hydrolysis, was unaffected by treatment with Endo Lys-C, in contrast to the results of others (12) (data not shown). Immunoblots were performed on the reaction mixtures, under non-reducing and reducing conditions, with polyclonal antisera to the active site- and carboxyl terminus-derived peptides (Fig. 7). Endo Lys-C had two effects on the 60-kDa doublet as follows: 1) both bands of the doublet were cleaved, resulting in a single band running at a slightly smaller molecular mass than the bottom band of the doublet; and 2) a time-dependent disappearance of the carboxyl-terminal epitope occurred. Antisera to the active site demonstrated that a single full-length chitinase remained after Endo Lys-C treatment under non-reducing conditions. Under reducing conditions a small fragment was removed, resulting in a slightly faster migration of the processed chitinase (Fig. 7, right top arrowhead). The precise epitope recognized by the carboxyl-terminal antisera, which demarcates the putative chitin-binding domain, contains a predicted Endo Lys-C site at Lys₅₀₉.

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**Fig. 5. Different forms of *P. gallinaceum* ookinete-produced chitinase and time course of expression.** a, aliquots of fractions 65 and 66 from Fig. 1e were subjected to 4–20% SDS-PAGE and transferred to nitrocellulose. The same blot was sequentially reacted with the carboxyl terminus antisera (C-term) (right panel), stripped, and then reacted with the active site (AS) antisera (left panel). b and c, Western immunoblot and determination of enzymatic activity were performed in a time course experiment. At 1.5 h after exflagellation and fertilization, the parasites are in the zygote stage. At 10 h, the intermediate form in which ookinetes are exiting the residual body, the retort stage, are present. Fully mature ookinetes are not seen until about 15 h after zygote formation. b, Western immunoblot of parasite proteins at various time points after zygote formation, using antisera directed against synthetic peptides derived from the catalytic active site and the carboxy-terminal domain. Parasite proteins were extracted with a mixture of protease inhibitors (see “Experimental Procedures”). An equal number of parasite equivalents were loaded into each lane or assayed for chitinase activity. Single arrowheads indicate proteins associated with peak 1 of chitinase activity (see Fig. 9); double arrowheads indicate proteins associated with peak 2 of chitinase activity. The thin arrow indicates the precursor of the protein doublet of 68-kDa indicated by the upper single arrowhead, as demonstrated by a Western immunoblot using antisera directed against the pro-enzyme domain (data not shown). Protein bands that do not increase in intensity during the course of ookinete development, and thus presumably are not chitinases, are not indicated with arrowheads. c, chitinase activity, as detected with 4-MU GlcNAc₃, as a function of time after zygote formation.
Ala<sup>510</sup>, consistent with the experimental findings. Cys<sup>506</sup>, at the amino terminus of the epitope, may form a disulfide bridge with the only cysteine downstream from it, Cys<sup>557</sup> (Fig. 3). Since Endo Lys-C converts the 60-kDa chitinase doublet to a slightly smaller single band, we determined the amino-terminal site of action of this protease. After treatment of rPgCHT1-NT1 with Endo Lys-C, the cleaved product was separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by Edman degradation. This analysis showed that Endo Lys-C cleaves rPgCHT1 on the carboxyl-terminal side of Lys<sup>86</sup>, just downstream from the NT2 cleavage site (Fig. 3). Immunoblot of Endo Lys-C-treated rPgCHT1 with the anti-carboxyl-terminal antiserum showed loss of the epitope (data not shown), similar to that found when native ookinete-produced 60-kDa chitinase was treated with Endo Lys-C (Fig. 7).

Expression of Recombinant PgCHT1—Numerous constructs of native codon-based PgCHT1, using different vectors expressed under a variety of temperature conditions and isopropyl-1-thio-β-D-galactopyranoside concentrations in E. coli host cells, did not produce more than ~5 μg of recombinant protein/liter-induced E. coli cells. Undetectable quantities of recombinant protein were obtained when attempting to express similar constructs in a well characterized Saccharomyces cerevisiae expression system (35). Because the A + T codon bias of this gene (70.6%) was suspected to be the primary barrier to producing recombinant protein, a synthetic PgCHT1 gene was constructed using E. coli-preferred codons and used as a PCR template for making the rPgCHT1-NT1 construct (see “Experimental Procedures”). Recombinant PgCHT1-NT1 (depicted schematically in Fig. 8c), expressed with a construct synthesized in E. coli-preferred codons and expressed in E. coli AD494 (DE3) cells, produced ~5–10 mg of recombinant protein/liter of induced cells, of which ~1–3% was soluble and active (data not shown). Chitinase activity was readily detectable in crude soluble extracts of the cells (data not shown). When the same constructs were expressed in E. coli BL21 (DE3) cells, no chitinase activity was detected, despite a comparable total quantity of protein produced (data not shown). Western immunoblot analysis of protein obtained by a Ni-NTA purification step demonstrated that the eluted, soluble proteins were >90% PgCHT1 (data not shown). Approximately 20% of the soluble protein in eluted fractions was rPgCHT1-NT1 of the predicted length; the rest were aggregated and truncated forms of rPgCHT1 (data not shown).

P. gallinaceum Chitinases Degrade Polymeric Chitinase and Have Identical Substrate Preferences and Reaction Product Profiles—A previous report demonstrated that several bands of chitinolytic activity were present in crude extracts and culture supernatants of P. gallinaceum ookinetes, as determined in a glycol chitin activity gel (14). rPgCHT1-NT1, whether or not treated with proteases, also degraded polymeric chitin in a glycol chitin activity gel (14). rPgCHT1-NT1, whether or not treated with proteases, also degraded polymeric chitin in a glycol chitin activity gel (14).
Endo Lys-C and enterokinase did in fact cleave the recombinant protein, Western immunoblot analysis performed before and after protease treatment demonstrated the appropriately sized cleavage products (data not shown).

To characterize the substrate specificity and reaction product profiles of *P. gallinaceum* chitinases, the activities of ookinetes were assayed with native chitin oligomers (Fig. 9) and 4-MU GlcNAc substrates (Fig. 10). *P. gallinaceum* ookinete-produced chitinase (peak 1) efficiently hydrolyzed 4-MU GlcNAc and 4-MU GlcNAc₄ substrates, while recombinant enzyme had no detectable activity against these substrates. The kinetics of Endo Lys-C-treated *P. gallinaceum* ookinete chitinase was determined to be 140 μM at pH 6.0 for 4-MU GlcNAc and 100 μM at 6.0 for 4-MU GlcNAc₄. The *Kₘ* values found for other recombinant and native chitinases are similar. For example, native and recombinant *Brugia malayi* chitinase has *Kₘ = 40 μM* (36).
and purified native *S. marcescens* ChiA and ChiB have $K_m = 40 \mu M$.

*P. gallinaceum* Chitinases Have Different pH Activity Profiles and Susceptibility to the Inhibitor Allosamidin—pH activity profiles were determined by microfluorimetry for peaks 1 and 2 of chitinase activity and for Endo Lys-C-treated rPgCHT1 (Fig. 11a) (peak 1 contains PgCHT1), using 4-MU GlcNAc$_3$ as substrate. Peak 2 chitinase activity had a broad pH optimum of pH 4.0–5.0. Peak 1 chitinase activity was optimal at pH 5.0. Similar to peak 1, rPgCHT1 also had a pH optimum of 5.0. This reproducible difference in pHe activity profiles suggests that the two peaks of chitinase activity are comprised of chitinases with different amino acids present in the catalytic sites of the enzymes and thus are products of different genes.

To confirm and extend the suggestion that *P. gallinaceum* ookinetes may secrete at least two chitinases derived from different genes, the sensitivity of each of the two peaks of chitinase activity and rPgCHT1 to the chitinase inhibitor allosamidin was determined (Fig. 11b). The IC$_{50}$ value estimated for peak 1 and rPgCHT1 were similar (7 and 12 \mu M, respectively). In contrast, the IC$_{50}$ value for peak 2 was 0.3 \mu M, about 30-fold less than that found for peak 1 or rPgCHT1. The allosamidin inhibition data provides additional evidence for a second *P. gallinaceum* chitinase gene and that peak 1 is the product of the *P.gCHT1* gene. The 1 and 0.1 \mu M concentrations of allosamidin used in previous studies to block oocyst development (12) far exceed the IC$_{50}$ values for both peaks of chitinase activity and would completely inhibit chitinase activity in ookinet extracts.

**DISCUSSION**

We report here the purification of a 60-kDa *P. gallinaceum* ookinite-secreted chitinase and characterize the gene, *PgCHT1*, encoding it. The experiments presented here identify at least two developmentally regulated chitinases expressed by *P. gallinaceum* ookinetes. Both are inhibited by allosamidin at concentrations far less than those used in *in vivo* studies for blocking ookinite penetration of the mosquito peritrophic membrane (12). Both are secreted and act as endochitinases, a property that would be expected of enzymes that allows the ookinite to penetrate and traverse the PM in the mosquito midgut.

At least two chitinase activities are separable by HPLC. The first, encoded by the gene, *PgCHT1*, was identified from peak 1 as a 60-kDa doublon (Fig. 1), which is composed of two forms of the protein, NT1 and NT2, which differ in size by 14 amino acids (Fig. 3). The NT1 form of PgCHT1, expressed as a recombinant protein in *E. coli*, has chitinase activity that is increased 73-fold by treatment with Endo Lys-C. The Endo Lys-C cleavage site is between eight and nine amino acid residues downstream from the amino terminus of the NT2 form, strongly suggesting that the NT2 form is an active chitinase. It is not clear if the NT1 form is fully active or needs to be converted further by parasite-produced proteases to the NT2 form for full activity. Whereas activity of the recombinant fusion protein rPgCHT1-NT1 is enhanced by Endo Lys-C, we found that Endo Lys-C has no effect on the activity of crude native ookinete-produced chitinase. One explanation of this discrepancy is that the 105-amino acid fusion partner of the recombinant fusion protein could be acting as the pro-enzyme domain does in the native produced gene product to inhibit enzyme activity and that the four amino acids remaining amino-terminal to the NT1 site remaining after enterokinase treatment prevent full activity of the enzyme. It is also possible that Endo Lys-C could inactivate the activity of some chitinases in crude ookinete culture supernatants while increasing the activity of other chitinases, leading to the finding of no overall change of chitinase activity of ookinete culture supernatants when treated by Endo Lys-C. The essential point is that we have demonstrated that the ookinite itself is capable of processing a pro-chitinase to a fully active form, and mosquito midgut proteases are not required for fully active ookinete-produced chitinase activity. This interpretation stands in contrast to previous models that hypothesize that the late trypsin expressed by the mosquito midgut more than 10 h after blood meal ingestion activate ookinete-produced chitinase (to increase the activity as much as 13-fold) for the purpose of optimizing the timing of mosquito midgut invasion (12, 16, 32). We have recently demonstrated that the *P. falciparum* chitinase homolog, PfCHT1, has no proenzyme domain and is a chitinase that does not require proteolytic activation for its enzymatic activity (31). The comparative activities of the NT1 and NT2 forms of PgCHT1 will be the focus of further study.

Peak 2 chitinase hydrolyzes 4-MU derivatives in a pattern similar to that produced by peak 1 and rPgCHT1 (data not shown). However, peak 2 chitinase has a distinct pH activity profile and is about 30-fold more sensitive to allosamidin (Fig. 11b) than rPgCHT1 and native peak 1 chitinase. Under reducing and denaturing conditions, a number of proteins, including a 35-kDa protein in peak 2, were identified by Western immunoblotting that increase in expression in parallel with an increased chitinase activity (Figs. 5 and 6). The 35-kDa protein reacts with antisera prepared from a peptide from the catalytic domain of PgCHT1, but not with antisera from a carboxy-terminal peptide of PgCHT1. Collectively, the different enzymatic properties and immunological reactivity of the peak 2 chitinase support the hypothesis that this chitinase is the product of a different gene, *PgCHT2*. Under non-reducing conditions, peak 2 chitinase migrates as a ~210-kDa protein. It is
not clear whether the complex would be composed solely of PgCHT2 protein subunits.

Other proteins (~30-kDa in peak 1 and ~160-kDa in peak 2) that cross-react with the active site antisera have not yet been further characterized, although these proteins also are stage-specifically expressed by ookinetes (Fig. 5). Additional chromatography of peak 2 suggests that the ~160-kDa protein in fact does have chitinase activity, as determined with the 4-MU GlcNAc3 substrate (data not shown). This latter observation is consistent with the possibility that these unidentified cross-reactive proteins are also chitinases. Experiments to identify and characterize the other chitinase proteins in peaks 1 and 2 at the molecular level are underway.

In the work presented here, we first identified a fragment of a putative P. falciparum chitinase. This fragment contains a catalytic domain but not a recognizable chitin binding domain; a stop codon appears at the end of the predicted catalytic domain. Work is in progress to characterize further the role of the P. falciparum chitinase gene, PfCHT1, in parasite invasion of the Anopheles midgut (31).

An important goal of research on the malaria parasite chitinases is to develop novel ways to interrupt malaria transmission. With the cloning of a full-length P. gallinaceum chitinase gene, recombinant chitinase can be synthesized and tested as a transmission-blocking vaccine candidate and characterized in parasite invasion in vivo. Preliminary sequence data for P. gallinaceum, P. falciparum, and P. knowlesi suggest that chitinases are safe and effective transmission-blocking vaccine candidates, avoiding potential autoimmune reactions. Determining three-dimensional structures of the Plasmodium chitinase domains may be possible and would provide the basis for rational design of a transmission-blocking drug. It may be possible to develop peptide chitinase inhibitors, the genes of which may be used for transforming mosquitoes (40, 41) to make them refractory to malaria transmission. Finally, we have initiated experiments to knock out the P. falciparum chitinase gene to determine whether its gene product is necessary for mosquito invasion or whether, perhaps, P. falciparum too has additional chitinase genes.

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