Excystation of *Eimeria tenella* Sporozoites Impaired by Antibody Recognizing Gametocyte/Oocyst Antigens GAM22 and GAM56

Juergen Kruecken,1*, Ralf J. Hosse,1 Aimdip N. Mouaf,2 Rolf Entzeroth,2 Stefan Bierbaum,1 Predrag Marinovski,1 Karolina Hain,1 Gisela Greif,3 and Frank Wunderlich1

Division of Molecular Parasitology and Biological and Medical Research Centre, Heinrich-Heine-University, Dusseldorf,1 Institute of Zoology, TU-Dresden, Dresden,2 and Animal Health Business Group, Research & Development, Bayer AG, Monheim,3 Germany

Received 10 August 2007/Accepted 14 November 2007

*Corresponding author. Mailing address: Division of Molecular Parasitology, Heinrich-Heine-University, Universitaetsstr. 1, 40225 Dusseldorf, Germany. Phone: 49-211-8114733. Fax: 49-211-8114734. E-mail: kruecken@uni-duesseldorf.de.

Eimeria tenella is the causative agent of coccidiosis in poultry. Infection of the chicken intestine begins with ingestion of sporulated oocysts releasing sporozocts, which in turn release invasive sporozoites. The monoclonal antibody E2E5 recognizes wall-forming body type II (WFBI) in gametocytes and the WFBI-derived inner wall of oocysts. Here we describe that this antibody also binds to the stieda body of sporocysts and significantly impairs in vitro excystation of sporozoites. Using affinity chromatography and protein sequence analysis, E2E5 is shown to recognize EtGAM56, the *E. tenella* ortholog of the *Eimeria maxima* gametocyte-specific GAM56 protein. In addition, this antibody was used to screen a genomic phage display library presenting *E. tenella* antigens as fusion proteins with the gene VIII product on the surfaces of phagemid particles and identified the novel 22-kDa histidine- and proline-rich protein Etgam22. The Etgam22 mRNA is expressed predominantly at the gametocyte stage, as detected by Northern blotting. Southern blot analysis in combination with data from the *E. tenella* genome project revealed that Etgam22 is an intronless multicopy gene, with approximately 12 to 22 copies in head-to-tail arrangement. Conspicuously, Etgam56 is also intronless and is localized adjacent to another gam56-like gene, Etgam59. Our data suggest that amplification is common for genes encoding oocyst wall proteins.

Coccidiosis in poultry is caused by protozoan parasites of the genus *Eimeria*. Worldwide economic losses due to these parasites have been estimated to exceed 1.2 billion U.S. dollars per annum (41). The most virulent species is *Eimeria tenella*, causing severe hemorrhagic enteritis by infection of the epithelium and submucosa of the ceca and, eventually, death of infected chickens (24).

*Eimeria* infections occur by ingestion of oocysts (24). In the intestine, oocysts release four sporozoites, each containing two sporozoites. After excystation, motile infective sporozoites actively enter cells in the epithelium of the cecum. Three rounds of asexual multiplication in the epithelium and submucosa are then followed by differentiation to sexual stages of micro- and macrogametocytes (23). After fertilization of macrogametes, a complex, two-layered wall is secreted around the young oocyst by excystation of wall-forming body type I and type II (WFBI and WFBII) (35). While the 10-nm-thick outer oocyst wall is built up by the contents of WFBI, the 90-nm inner oocyst wall is composed mainly of glycoproteins that were stored in WFBI and WFBII (35). The oocyst displays a remarkable rigidity and protects the parasite from several physically and chemically adverse influences, such as commonly used disinfectants (34). A potential use of gametocyte antigens involved in formation of the oocyst wall as protective transmission-blocking vaccines has been described for *Eimeria maxima* (2, 4, 25, 38–40, 46).

The formation of oocyst and sporocyst walls and sporozoite excystation are rather complex processes that we are just beginning to understand. Only a few WFBI-localized glycoproteins have been characterized for *E. tenella* (10) and for *E. maxima*. They have been shown to undergo site-specific proteolysis before incorporation into the mature oocyst wall (1, 3, 4, 5). Moreover, there is compelling evidence for the occurrence of cross-linking of these tyrosine-rich proteins. This process of sclerotization involves the formation of dityrosine bonds as well as the emergence of covalent bonds between proteins by peroxidase-mediated mechanisms involving 1,3,4-dihydroxyphenylalanine (DOPA). Incidentally, sclerotization of the oocyst wall appears to be comparable to other hardening processes in extracellular matrices, such as insect and nematode cuticles, yeast cell walls, mussel byssal threads, and sea urchin fertilization membranes (3).

In *E. tenella*, the WFBI of macrogametocytes and the inner oocyst wall can be labeled specifically by the monoclonal antibody E2E5 (26). This antibody recognizes a complex, developmentally regulated pattern of protein bands in Western blot analysis (26). Here we show that E2E5 recognizes proteins encoded by at least two different genes and impairs sporozoite excystation.

**MATERIALS AND METHODS**

*Animals, parasites, and infections.* The strain *E. tenella* VT-2 was used throughout all experiments. Male chickens of Leghorn type strain LSL (Josef Brinkschulte GmbH, Senden, Germany) were infected with 15,000 oocysts. For preparation of oocysts, infected chickens were killed, and the contents of the cecum were flushed out with 2% potassium dichromate solution. Sporulation of oocysts was completed after they were stirred in 2% potassium dichromate at 28°C for 48 h.

*Cell culture.* The hybridoma cell lines E1D8 and E2E5 were previously described for specifically recognize antigens in WFBI and WFBII, respectively (26).
Hybridomas and the human T-cell lymphoma cell line Jurkat were cultivated in RPMI 1640 supplemented with 10% fetal calf serum at 37°C, 5% CO2, and 100% humidity. For most experiments, supernatants were concentrated 50-fold using Vivaspin concentrators (Sartorius AG, Göttingen, Germany) with a 100-kDa cutoff.

**Immunofluorescence.** Reactivity of E2E5 to intracellular E. tenella stages was analyzed as described previously (26). Briefly, semithin sections of LR-White-embedded ceca from E. tenella-infected chickens were probed with unidiluted hybridoma culture supernatants before detection with fluorescein isothiocyanate (FITC)-coupled anti-mouse immunoglobulin G (IgG) (for E2E5) or rhodamine-coupled anti-mouse immunoglobulin M (IgM) (for E1D8) antibody (Sigma, Germany). Sections were examined using a Zeiss Axioscope 2 microscope.

Oocytes were vortexed in the presence of glass beads until rupturing of most walls of oocytes and sporocytes. After centrifugation, the pellet was resuspended in methanol (−20°C), incubated for 10 min at −20°C, washed in phosphate-buffered saline (PBS), and subjected to immunofluorescence microscopy as described recently (18). Oocytes and sporocytes were treated with 0.1% Triton X-100 in PBS for 10 min, blocked for 1 h in PBS-1% bovine serum albumin (BSA), incubated with a 1:40 dilution of concentrated E2E5 supernatant for 2 h, and finally visualized with a 1:100 dilution of a secondary goat anti-mouse antibody coupled to Alexa Fluor 488 (Molecular Probes, Karlsruhe, Germany).

**Excystation assay.** Freshly sporulated oocytes were ruptured in a glass Teflon potter. Free sporocytes were incubated overnight at 4°C in PBS containing 10 µg/ml enoxafolin (Bayer; Trier, Bayer, Leverkusen, Germany). Sporocytes were then collected by centrifugation and resuspended in 20 ml PBS containing 2.5 µg/ml trypsin before adding 1 ml of chicken bile. Aliquots of 250 µl were incubated in the presence of either 12.5 µl E2E5 concentrated 50-fold or control supernatants at 4°C and 5% CO2 for 5 h. Numbers of sporocytes before excystation and of free sporozoites after excystation were counted in a Neubauer chamber. Statistical comparisons between groups were done using paired Student’s t-test.

**Affinity chromatography and Edman degradation.** E. tenella gametocytes were purified as described recently (26). Proteins were solubilized with 0.5% Triton X-100 PBS containing 1 mM phenylmethylsulfonyl fluoride. Columns containing 4 ml protein A-Sepharose CL-4B covalently cross-linked to E2E5 (Amersham Schwalbach, Germany). The sequence of the NH2 terminus of the protein was determined using Edman degradation at the University of Gent (Belgium).

**Construction of phage display library.** Eimeria tenella sporozoites were vortexed in the presence of glass beads until rupturing of most walls of sporozoites and sporocytes. After centrifugation, the pellet was resuspended in methanol (−20°C), incubated for 10 min at −20°C, washed in phosphate-buffered saline (PBS), and subjected to immunofluorescence microscopy as described (18). Oocytes and sporocytes were treated with 0.1% Triton X-100 in PBS for 10 min, blocked for 1 h in PBS-1% bovine serum albumin (BSA), incubated with a 1:40 dilution of concentrated E2E5 supernatant for 2 h, and finally visualized with a 1:100 dilution of a secondary goat anti-mouse antibody coupled to Alexa Fluor 488 (Molecular Probes, Karlsruhe, Germany).

**Screening of phage display library.** In order to identify phage clones expressing fusion proteins reacting with E2E5, the latter was immobilized on magnetic pan-mouse IgG Dynabeads (Invitrogen) according to the manufacturer’s instructions. All incubations containing Dynabeads were carried out at 4°C with rotation. After being blocked with PBS-0.1% BSA, beads were collected using a Dynal MPC-S magnet (Deutsche Dynal GmbH). For every screening round, two parallel binding reaction mixtures containing 2 × 1010 control or E2E5-coupled beads and 200 µl phageadins in a final volume of 400 µl PBS-0.1% BSA were set up. After overnight binding, beads were washed 10 times for 5 min each with 2 ml PBS-0.1% BSA and once for 15 min with 150 mM NaCl-50 mM sodium citrate (pH 4.5) before phageadins were eluted with 400 µl 150 mM NaCl-50 mM sodium citrate (pH 1.8). After neutralization with 40 µl 2 M Tris-Cl (pH 8.6), the phageadins were used to reinfet E. coli TG1 cells, titrated, and amplified before use in consecutive screening rounds.

**RNA and protein isolation.** For reverse transcription-PCR (RT-PCR), DNA-free total RNAs from ruptured oocytes and chicken ceca were isolated using an RNAqueous Maxi kit (Ambion, Austin, TX, USA). All PCR reactions were incubated without AMV reverse transcriptase or without RNA. PCR mixtures contained 5 µl of the products, 0.4 µl of each primer, 10 µl of both oligo(dT)15 (Pharmacia, Freiburg, Germany) and 5 µl Expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 50 µl high-fidelity buffer. The enzyme was added during the first annealing step to achieve a hot start. Synthesis of cDNA was performed using the M-MLV reverse transcriptase by adding 2 µg total RNA, 0.5 unit RNasin (Promega, Madison, WI), 2 mM of each dNTP, 50 mM Tris-HCl, pH 9.0, 0.5 mM each primer and 1 µl of cDNA, 10 µl Q solution (Qiagen, Hilden, Germany), and 1 µl of cDNA as the template, as described for 3′-RACE, using the PCR anchor primer and the primer 5′-TAGGAGCTATCTCTAGACCTAGTCGTTTCT-3′ and 5′ U expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 50 µl high-fidelity buffer. The enzyme was added during the first annealing step to achieve a hot start. Synthesis of cDNA was performed using the M-MLV reverse transcriptase by adding 2 µg total RNA, 0.5 unit RNasin (Promega, Madison, WI), 2 mM of each dNTP, 50 mM Tris-HCl, pH 9.0, 0.5 mM each primer and 1 µl of cDNA, 10 µl Q solution (Qiagen, Hilden, Germany), and 1 µl of cDNA as the template, as described for 3′-RACE, using the PCR anchor primer and the primer 5′-TAGGAGCTATCTCTAGACCTAGTCGTTTCT-3′ and 5′ U expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 50 µl high-fidelity buffer. The enzyme was added during the first annealing step to achieve a hot start. Synthesis of cDNA was performed using the M-MLV reverse transcriptase by adding 2 µg total RNA, 0.5 unit RNasin (Promega, Madison, WI), 2 mM of each dNTP, 50 mM Tris-HCl, pH 9.0, 0.5 mM each primer and 1 µl of cDNA, 10 µl Q solution (Qiagen, Hilden, Germany), and 1 µl of cDNA as the template, as described for 3′-RACE, using the PCR anchor primer and the primer 5′-TAGGAGCTATCTCTAGACCTAGTCGTTTCT-3′ and 5′ U expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 50 µl high-fidelity buffer. The enzyme was added during the first annealing step to achieve a hot start. Synthesis of cDNA was performed using the M-MLV reverse transcriptase by adding 2 µg total RNA, 0.5 unit RNasin (Promega, Madison, WI), 2 mM of each dNTP, 50 mM Tris-HCl, pH 9.0, 0.5 mM each primer and 1 µl of cDNA, 10 µl Q solution (Qiagen, Hilden, Germany), and 1 µl of cDNA as the template, as described for 3′-RACE, using the PCR anchor primer and the primer 5′-TAGGAGCTATCTCTAGACCTAGTCGTTTCT-3′ and 5′ U expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 50 µl high-fidelity buffer. The enzyme was added during the first annealing step to achieve a hot start.
AGAAACGCTGTGCACTTC-3' and 5'-CGGACGAAAATGCGCGAGCGAG-3'. The reaction mixtures were denatured for 2 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 63°C, and 1 min at 72°C. A final extension for 10 min at 72°C completed the reactions.

Amplification of full-length Etgam22 cDNA was carried out with 5 μl cDNA from infected ceca at 137 h postinfection (p.i.). Reactions were performed as described above, using the primers 5'-CAGGACGCCAAATAAAAATCAAAGCCTATCA-3' and 5'-TGACCGGTGTTGTATCTCGTAAC-3'.

Northern blotting. RNAs (20 μg) were glyoxylated, separated in agarose gels, transferred to Hybond-N membranes (Amersham), and hybridized as described recently (42).

In vitro translation. Coupled in vitro transcription and translation were performed using the TNT quick coupled transcription/translation system (Promega). In vitro translation products were separated by SDS-PAGE and subjected to Western blotting using the E2E5 antibody.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number CS000361.

RESULTS

In vitro inhibition of excystation. The monoclonal antibody E2E5 recognized antigens in the WFBII of gametocytes and in the inner oocyst wall of E. tenella (Fig. 1A to C), thus confirming our previous results (26). In addition, we show here that E2E5 reacts with the apical stieda body in ruptured but not intact sporocysts (Fig. 1D). This indicates that the epitope is not accessible for the E2E5 antibody from the outside.

Since the apical stieda body of sporocysts is involved in the excystation process of sporozoites, we also tried to inhibit in vitro excystation of E. tenella sporozoites from the sporocytes with E2E5. The oocyst walls were mechanically disrupted, and excystation was induced by incubation of free sporocysts with trypsin and bile at 41.5°C for 5 h. In these control experiments, between 48% and 77% of all possibly available sporozoites were released from sporocysts. The addition of supernatants from E2E5 significantly decreased the release of sporozoites, by about 50% (Fig. 1E). In contrast, neither supernatants from E1D8 hybridoma cells nor supernatants from Jurkat cells, with or without supplementation with an irrelevant IgG2a antibody (anti-V5 tag), had any significant effect on excystation.

E2E5 recognizes a complex pattern of proteins in the different developmental stages of E. tenella (23). In order to identify genes encoding the corresponding proteins, we used two complementary approaches. First, we characterized the largest of the proteins by affinity chromatography and Edman degradation. Second, we used phage display to select cDNAs encoding proteins recognized by E2E5.

Purification and identification of EmGAM56 from gametocytes. Young gametocytes express only a single protein, of about 51 kDa, that is recognized by E2E5. Affinity chromatography was performed with E2E5 coupled to protein A agarose. The protein fraction eluted from the column was separated by SDS-PAGE and either silver stained (Fig. 2) or blotted on a polyvinylidene difluoride membrane for protein sequencing. Edman degradation of the N\textsubscript{\textalpha} terminus resulted in the sequence VPTTVENTVHPYIHMHGQYQGPRYAYMG. Database screening using this sequence revealed a 78% identity of amino acids 3 to 21 of this sequence to the GAM56 protein of Eimeria maxima (EmGAM56) (1, 5). Moreover, 20 of the first 21 amino acids in this sequence can be deduced from the sequence of a genomic contig deposited in the E. tenella database. An intronless open reading frame of 374 amino acids encoding a 54-kDa protein can be deduced from the corresponding region of the genomic sequence dev_EIMER_contig_00030093-eimer-679b04.p1ka (data not shown).

This putative protein has 63% identity and 73% similarity to EmGAM56, as revealed by BLAST analysis. Since both EmGAM56 and the 54-kDa protein recognized by E2E5 are expressed selectively in the macrogametocyte stage and antibodies to EmGAM56 also selectively recognize WFBII, we...
propose that the 54-kDa protein is the *E. tenella* ortholog to GAM56 of *E. maxima* and should be designated EtGAM56.

Interestingly, a second GAM56-like protein, of 59 kDa, is encoded on the same contig and in the same orientation, about 2.3 kb upstream of EtGAM56. We designate this intronless gene *Etgam59*, although we have not shown gametocyte-specific expression of *Etgam59* yet. Figure 3 shows a multiple sequence alignment of both deduced *E. tenella* proteins with the GAM56 sequence of *E. maxima*. The sequences of all three GAM56-like proteins are rich in the amino acids Ala, Pro, Thr, and Ser. Moreover, all of these proteins contain a Tyr-rich domain. EmGAM56 is known to be present in the WFBII, the inner oocyst wall, and the apical part of the sporocyst, and this fits perfectly well to our data from immunofluorescence and immunoelectron microscopy presented in Fig. 1 and to the data of Mouafo et al. (26). Remarkably, EmGAM56 has been shown to be proteolytically cleaved during oocyst formation into mature polypeptides of 33 kDa and 12 kDa (3). Indeed, the protein band corresponding to EtGAM56 disappears in unsporulated oocysts, and a strong band of slightly larger than 30 kDa appears (26).

**Cloning of EtGAM22 by phage display.** In addition to EtGAM56 and its 33-kDa proteolytic fragment, E2E5 recognizes two smaller proteins, of approximately 23 kDa and 25 kDa, in unsporulated oocysts and an approximately 80-kDa protein in sporulated oocysts (26). In order to identify additional proteins recognized by E2E5, a genomic phage display library presenting recombinant *E. tenella* proteins as fusions with the gene VIII product of filamentous phage on the surfaces of phagemid particles was constructed. After four rounds of library parking against E2E5 coupled to Dynabeads, enrichment of a clone containing an insert of 145 bp was observed (data not shown). Western blot analysis clearly revealed that this phage clone, clone A17, expresses a recombinant gene VIII product fusion protein that is recognized by E2E5, while other clones were consistently negative (Fig. 4).

Using 5′- and 3′-RACE PCR, we cloned the complete open reading frame of the corresponding mRNA. Figure 5 shows the cDNA sequence of Etgam22 and the deduced protein sequence. The EtGAM22 protein has a length of 198 amino acids, a molecular mass of 22.8 kDa, and a predicted pI of 6.8. At the NH₂ terminus, a signal peptide for cotranslational transport into the endoplasmic reticulum (ER) is predicted by the SignalP 3.0 program (6). The amino acid composition of the mature EtGAM22 protein without a signal peptide is very unusual, since only four amino acids, His (25.7%), Pro (19%), Gln (8.4%), and Ala (6.1%), account for nearly 60% of all residues. In particular, an extremely His- and Pro-rich domain between Pro⁷³ and His¹⁸⁸ can be identified. This partially resembles the case for EmGAM56, which contains 12.8% Pro and 8% Ala residues. In contrast to EtGAM22, however, EmGAM56 contains only 0.6% His and 2.9% Gln residues, whereas EtGAM22 contains no Tyr-rich domain characteristic of other gametocyte-specific proteins, such as EmGAM56 and EmGAM82 (3). The sequence of the original phage clone A17 containing the E2E5 epitope is completely localized within this His/Pro-rich domain. Moreover, there are putative O glycosylation sites at Thr⁷⁴, Thr⁸⁰, and Thr⁸¹, as predicted by NetOGlyc 3.1.

**Genomic organization of EtGAM22.** BLAST analysis of the sequences deposited by the *E. tenella* genome project identified several perfect matches showing that the Etgam22 gene—like Emgam56, Etgam56, and Etgam59—does not contain any introns. Conspicuously, there are two contigs (dev_EIMER_contig_00003072-eimerbac28g88fb07.q1k [data not shown] and dev_EIMER_contig_00010656-eimer-437a12.q1k) with two identical head-to-tail repeats of the Etgam22 gene. One of these contigs is drawn schematically in Fig. 6A. In addition, the intergenic regions between the copies are also nearly perfectly conserved (99% identity), indicating that the promoter regions are virtually identical. In order to demonstrate that Etgam22 is indeed a multigene copy, we performed genomic Southern blot analysis using six different restriction enzymes (Fig. 6B). With the exception of DraI, all enzymes produced one intensive band and one or two faint bands, which is in accordance with several virtually identical head-to-tail copies of Etgam22. In particular, it should be mentioned that the restriction sites for all the enzymes used here are located outside the Etgam22 open reading frame, indicating a high degree of conservation even in the noncoding sequences of the cluster. The faint bands represent unique fragments from the end of the Etgam22 cluster. We used two complementary approaches to estimate the Etgam22 copy number in the *E. tenella* genome. The genomic DNA digested with BglI and MvaI (Fig. 6B) yielded two bands, i.e., a faint band representing a single copy located at the end of the Etgam22 cluster and a strong band due to hybridization to the other members of the cluster. After correction for different overlaps of internal and flanking Etgam22 copies with the hybridization probe, it should be possible to calculate the copy number from the ratio of band intensities. Our results indicate that there are 14 times more hybridization targets in the strong than in the faint BglI band and 11 times more targets in the strong than in the faint MvaI band. Therefore, there are between 12 and 15 copies of Etgam22 in the genome.

For an independent approach to estimate the copy number...
of Etgam22, we used the single band produced in Southern blots after digestion with DraI. Genomic DNA from E. tenella was digested with DraI and mixed with XhoI-restricted Etgam22 in the plasmid vector pcDNA3.1 (6,629 bp). Twofold serial dilutions of this mixture were analyzed by Southern hybridization (Fig. 6C). After densitometric evaluation of band intensities, the optical densities of the plasmid bands were evaluated using a phosphorimager and then plotted against the copy number per lane. Using this standard curve, the number of Etgam22 copies in genomic DNA per lane was calculated from the band intensities for genomic DNA. Finally, the Etgam22 copy number per genome was calculated to be 19/11006, assuming a genome size of 60 Mbp and using only those genomic DNA bands with intensities within the range of the standard curve. Presumably, this is a more accurate value since the limited linear range of X-ray film compared to that of a phosphorimager may lead to underestimation of strong band intensities.

Gametocyte-specific expression of EtGAM22. RT-PCR analysis showed that the Etgam22 mRNA is not expressed in the

FIG. 3. Sequence alignment of GAM56-like proteins from E. tenella and E. maxima. Amino acids showing identity to the sequence obtained from Edman sequencing of the NH2 terminus are highlighted in gray. Invariable amino acid positions are marked with asterisks, and substitutions rated conservative and semiconservative by ClustalW using the GONNET 250 matrix are marked with colons and periods, respectively.
early stage of infection, at 72 h p.i., whereas it is readily detectable at 137 h p.i. and sporulated oocysts (Fig. 7A). However, expression levels in these stages differ widely, as revealed by Northern blot analysis (Fig. 7B). Thus, Et\textsuperscript{gam22} was not detectable in sporulated oocysts, while a faint hybridization signal was obtained from RNAs of gametocytes at 138 h p.i. Expression of Et\textsuperscript{gam22} mRNA then increases dramatically at 148 h p.i. and 168 h p.i., when mature gametocytes and a mixture of gametocytes and unsporulated oocysts, respectively, are present in the ceca. Expression of E\textit{tgam}56 mRNA was already very weakly detectable at 132 h p.i. and showed maximum expression between 144 h p.i. and 168 h p.i.

We also extracted proteins from the same tissue samples and analyzed the expression of proteins by using the antibody E2E5 (Fig. 7B). Weak expression of the approximately 60-kDa band could be detected as early as 132 h p.i. Bands of about 33 kDa and 25 kDa did not appear before 168 h p.i. and were still detectable in sporulated oocysts. In this context, it is noteworthy that in \textit{E. maxima}, the ortholog EmGAM56 is cleaved into a 33-kDa polypeptide during oocyst formation (3). The 33-kDa protein in Fig. 7B is therefore assumed to represent the proteolytic fragment of EtGAM56. In accordance with our previous results, a 25-kDa band was also observed at 168 h p.i. However, we could not reproduce the weak 23-kDa band detected previously in purified unsporulated oocysts (26). Since in vitro translation of Et\textit{gam22} cDNA also produced a polypeptide of about 25 kDa (Fig. 7C), the 25-kDa band at 168 h p.i. most likely corresponds to EtGAM22. This view is also consistent with the fact that processing of EmGAM56 does not give rise to polypeptide fragments in the size range of 20 to 25 kDa (3).

**DISCUSSION**

The WFBII in gametocytes provides essential components for the rigid and impermeable walls of oocysts, whose formation is a critical step in the life cycle of \textit{Eimeria} parasites, which...
rely exclusively on the fecal/oral route for infection of new hosts. Using the monoclonal antibody E2E5, we describe the *E. tenella* GAM56 ortholog and confirm its proteolytic processing during oocyst wall formation. Moreover, we identify a novel type of small gametocyte-specific protein, EtGAM22, which also appears to be a structural component of the oocyst wall.

The genes encoding EtGAM22 and EtGAM56, identified here for *E. tenella*, and the previously characterized genes for the WFBII-localized proteins EmGAM56 and EmGAM82 in *E. maxima* have several characteristics in common. All of them are expressed specifically in gametocytes. Moreover, there is a remarkable absence of introns in these genes. Although Emgam56 was described to be a single-copy gene (5), the data presented here indicate the presence of two closely related genes, Etgam56 and Etgam59, in the genome of *E. tenella*. Etgam22 is the first multicopy gene described for *Eimeria* species, and its extraordinarily high copy number and the extremely conserved sequence between the copies suggest that Etgam22 has a particularly important role in oocyst wall formation. The high degree of conservation indicates that amplification of the Etgam22 gene was a very recent evolutionary event or that the sequences of the copies are frequently equalized by unequal crossover or gene conversion, which prevents
sequence diversification, as previously described for multigene families, including rRNA (33) and histone (30, 32) genes. Apparently, such mechanisms do not act on gam56-like genes.

Among the apicomplexa, low-copy-number head-to-tail clusters of homologous intronless genes have been described for some of the T. gondii SAG genes that encode glycosylphosphatidylinositol-coupled surface antigens expressed by the invasive parasite stages (11, 21, 36). Sequence similarity and expression levels in different developmental stages vary widely between family members in the same cluster, and intergenic regions are usually not conserved, with the exception of those of SAG5B and SAG5C (36). In contrast, the intergenic regions in the Etgam22 gene cluster appear to be highly conserved, as far as can be deduced from the few sequenced repeats and the conservation of restriction sites, although we do not yet know whether all genes in the cluster are transcriptionally active. Expression of both Etgam56 and Etgam22 mRNAs is very high, since exposure times of Northern blots of as short as 2.5 h and 4 h, respectively, were sufficient to produce clearly detectable bands, despite the fact that the RNAs were not isolated from pure parasites but from infected ceca and would therefore contain large amounts of chicken RNA. It is therefore tempting to speculate that amplification of the Etgam22 gene copy number might be a mechanism to allow the production of large amounts of RNA within a very short period, as previously described for histone genes, which are highly expressed only during a short phase of the cell cycle (22). Despite the high level of Etgam22 mRNA, however, the protein band detected in Western blots is surprisingly quite faint. There are several possible explanations for this observation. First, the reactivity of the E2E5 antibody might be better for EtGAM56 than for EtGAM22, or extraction of EtGAM56 from the tissue might be easier than that of EtGAM22. Second, translation of Etgam22 mRNA might be inefficient, with only low levels of protein being produced. Third, translation of Etgam22 mRNA may be developmentally regulated and may not occur in macrogametocytes but in oocytes after fusion of gametes or during sporulation. This view is also supported by the fact that the EtGAM22 protein is not detectable at 144 h p.i., when transcript levels are maximal, although transcription starts at least 6 h earlier. However, the protein becomes detectable at 168 h p.i., when the first unsporulated oocysts appear in the cecum, as revealed by the beginning of proteolytic processing of EtGAM56.

At the protein level, similarity between EtGAM22 and EtGAM56 manifests itself as cross-reactivity with E2E5, as the presence of putative N-glycosylation sites, and as NH2-terminal signal peptides. The presence of a signal peptide and the fact that, in macrogametocytes, E2E5 exclusively recognizes WFBI, i.e., specialized regions within the rough ER (9), suggest that EtGAM22—like EmGAM56—is transported to the WFBI and participates in formation of the inner oocyst wall and/or the stieda body. For E. maxima, a GAM56-specific monoclonal antibody has been reported to recognize the WFBI in macrogametocytes, the ooyct wall, the outer sporocyst wall, and the stieda body (5). Since this pattern largely overlaps with the pattern of E2E5 labeling in E. tenella, a monoclonal antibody against EtGAM22 that does not cross-react with EtGAM56 will be necessary to evaluate the exact localization of EtGAM22.

Other His-rich proteins are known for parasites belonging to the apicomplexa, with the histidine-rich protein of Plasmodium falciparum (78% His residues) (12) displaying the most exceptional composition. However, the low sequence complexities of these proteins and EtGAM22 do not permit a phylogenetic analysis. Indeed, those His-rich proteins for which a function is known are surely not homologous to EtGAM22. For instance, the knob-associated histidine-rich protein of Plasmodium falciparum is involved in the interaction of parasite proteins in knobs on the erythrocyte surface with the host cell cytoskeleton (27, 29), whereas the histidine-rich protein 2 has been implicated in hemozoin formation of P. falciparum (28). In contrast, BLAST comparison of EtGAM22 with proteins predicted from the Toxoplasma gondii genome reveals the presence of at least five proteins with NH2-terminal signal peptides which are relatively rich in proline and histidine and have lengths in the range of 117 to 269 amino acids. Although there is not yet anything known about the function or developmental expression pattern of these proteins, it is tempting to speculate that some of them represent extracellular structural proteins, such as many proline- or hydroxyproline-rich glycoproteins in plant cell walls (15, 16) or histidine-rich proteins in Hydra nematocysts (17). Cross-linking of EmGAM56- and EmGAM82-derived peptides via dopamine has been shown in the oocyst wall (3), and His-rich proteins such as EtGAM22 might also be involved in stabilizing extracellular structures via cross-links between His and catechols, as described for insect cuticles (8, 14, 43).

The in vitro exocystation inhibition assay presented here shows that the antibody E2E5 can significantly interfere with parasite development. Remarkably, vaccination with a preparation of native gametocyte antigens enriched for GAM56, GAM82, and GAM230, which are all involved in cell forming in E. maxima, has been shown to convey at least partial protection against homologous challenge (25, 39, 46). Since EtGAM22 represents a new family of oocyst wall proteins, it might be a useful supplement to a protective gametocyte-specific cocktail vaccine.

ACKNOWLEDGMENTS

We thank Carsten Angenendt for valuable technical assistance. Genomic data were provided by The Institute for Genomic Research (supported by NIH grant AI05093) and by the Sanger Center (Wellcome Trust). Expressed sequence tag sequences were generated by Washington University (NIH grant 1R01AI045806-01A1).

REFERENCES

1. Belli, S. I., M. Lee, P. Thebo, M. G. Wallace, B. Schwartzburd, and N. C. Smith. 2002. Biochemical characterisation of the 56 and 82 kDa immunodominant gametocyte antigens from Eimeria maxima. Int. J. Parasitol. 32: 805–816.
2. Belli, S. I., K. Mai, C. D. Skene, M. T. Glesson, D. M. Witcombe, M. Katrib, A. Finger, M. G. Wallace, and N. C. Smith. 2004. Characterisation of the antigenic and immunogenic properties of bacterially expressed, sexual stage antigens of the coccidian parasite Eimeria maxima. Vaccine 22:4316–4325.
3. Belli, S. I., M. G. Wallace, C. Luxford, M. J. Davies, and N. C. Smith. 2003. Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite Eimeria maxima. Eurakroy. Cell 22:456–464.
4. Belli, S. I., M. G. Wallace, and N. C. Smith. 2003. Cloning and characterisation of the 82 kDa tyrosine-rich sexual stage glycoprotein, GAM82, and its role in oocyst wall formation in the apicomplexan parasite Eimeria maxima. Gene 307:201–212.
5. Belli, S. I., D. Witcombe, M. G. Wallace, and N. C. Smith. 2002. Functional
IMPAIRED EXCYSTATION OF EIMERIA TENELLA

211

is anchored to the actin-spectrin junction and kno-associated histidine-rich protein in the erythrocyte skeleton. Mol. Biochem. Parasitol. 108:237–247.

28. Pandey, A. V., V. K. Babbarwal, J. N. Okoyeh, R. M. Joshi, S. K. Purii, R. L. Singh, and V. S. Chauhan. 2003. Hemozoin formation in malaria: a two-step process involving histidine-rich proteins and lipids. Biochem. Biophys. Res. Commun. 308:736–743.

29. Pei, X., X. An, X. Guo, M. Tarnawski, R. Coppell, and N. Mohandas. 2005. Structural and functional studies of interaction between Plasmodium falciparum knob-associated histidine-rich protein (KaHRP) and erythrocyte spectrin. J. Biol. Chem. 280:31166–31171.

30. Piontkivska, H. A., P. Rooney, and M. Nei. 2002. Purifying selection and birth-and-death evolution in the histone H4 gene family. Mol. Biol. Evol. 19:689–697.

31. Pittilo, R. M., and S. J. Ball. 1980. The ultrastructural development of the oocyst wall of Eimeria maxima. Parasitology 81:115–122.

32. Rooney, A. P., and H. Piontkivska, and M. Nei. 2002. Molecular evolution of the nonrandomly repeated genes of the histone 3 multigene family. Mol. Biol. Evol. 19:68–75.

33. Spano, F., I. Ricci, M. Di Cristina, A. Possenti, M. Tinti, N. Dendouga, S. Tomavo, and A. Crescanti. 2002. The SAG5 locus of Toxoplasma gondii encodes three novel proteins belonging to the SAG1 family of surface antigens. Int. J. Parasitol. 32:121–131.

34. Stotish, R. L., C. C. Wang, and M. Meyenhofer. 1978. Structure and composition of the oocyst wall of Eimeria tenella. J. Parasitol. 64:1074–1081.

35. Wallach, M. 1997. The importance of transmission-blocking immunity in the control of infections by apicomplexan parasites. Int. J. Parasitol. 27:1159–1167.

36. Wallach, M. 2002. The development of a novel vaccine against coccidiosis. World Poultry 19:24–26.

37. Wallach, M. G., D. Mencher, S. Yaras, G. Pillmer, A. Halabi, and T. Jugat. 1989. Eimeria maxima: identification of gametocyte protein antigens. Exp. Parasitol. 68:49–56.

38. Williams, R. B., W. W. Carlyle, D. R. Bond, and I. A. Brown. 1999. The efficacy and economic benefits of Paraxon, a live attenuated anticoccidial vaccine, in commercial trials with standard broiler chickens in the United Kingdom. Int. J. Parasitol. 29:341–355.

39. Wunderlich, F., M. A. Dkhil, L. I. Mehner, J. V. Braun, M. El Khadragy, E. Borsch, D. Hermens, W. P. Benten, K. Pfeffer, H. Mossmann, and J. Stotish. 2005. Testosterone responsiveness of spleen and liver in female lymphocytic beta receptor-deficient mice resistant to blood-stage malaria. Microbes Infect. 7:599–609.

40. Xu, R. D., X. Huang, T. L. Hopkins, and K. J. Kramer. 1997. Catecholamine and histidyl protein cross-linked structures in selenitized insect cuticle. Insect Biochem. Mol. Biol. 27:101–109.

41. Zdobnov, E. M., and R. Apweiler. 2001. InterProScan—an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17:447–448.

42. Zhang, L., K. Jacobsson, K. Strom, M. Lindberg, and L. Frykberg. 2001. InterProScan—an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17:447–448.

43. Ziemiecki, R., J. Karamon, T. Cencek, E. Gornowicz, A. Skoracki, and U. Ashash. 2005. Prevention of broiler chick coccidiosis using the inactivated subunit vaccine CoccAbic. Bull. Vet. Inst. Puawy 49:299–302.