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*

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Received 16 December 2002/Accepted 27 February 2003

*Eimeria maxima* is an intestinal parasite of chickens and is one of the causative agents of coccidiosis, contributing to costs on the order of billions of dollars per year to the poultry meat industry (34, 37). The life cycle of *Eimeria* includes asexual and sexual stages of development that lead to the formation of the infective form of the parasite, the oocyst (23). Prior to excretion, the oocyst is encapsulated by a hard barrier, the oocyst wall, which protects the parasite from the harsh external environment. Once excreted from the host, the oocyst develops further (sporulation) and is passed onto the next host via the fecal-oral route.

The oocyst wall of apicomplexan parasites protects them from the harsh external environment, preserving their survival prior to transmission to the next host. If oocyst wall formation could be disrupted, then logically, the cycle of disease transmission could be stopped, and strategies to control infection by several organisms of medical and veterinary importance such as *Eimeria*, *Plasmodium*, *Toxoplasma*, *Cyclospora*, and *Neospora* could be developed. Here, we show that two tyrosine-rich precursor glycoproteins, gam56 and gam82, found in specialized organelles (wall-forming bodies) in the sexual stage (macrogamete) of *Eimeria maxima* are proteolytically processed into smaller glycoproteins, which are then incorporated into the developing oocyst wall. The identification of high concentrations of dityrosine and 3,4-dihydroxyphenylalanine (DOPA) in oocyst extracts by high-pressure liquid chromatography, together with the detection of a UV autofluorescence in intact oocysts, implicates dityrosine- and possibly DOPA-protein cross-links in oocyst wall hardening. In addition, the identification of peroxidase activity in the wall-forming bodies of macrogametes supports the hypothesis that dityrosine- and DOPA-mediated cross-linking might be an enzyme-catalyzed event. As such, the mechanism of oocyst wall formation in *Eimeria*, is analogous to the underlying mechanisms involved in the stabilization of extracellular matrices in a number of organisms, widely distributed in nature, including insect resins, nematode cuticles, yeast cell walls, mussel byssal threads, and sea urchin fertilization membranes.

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*MATERIALS AND METHODS*

**Animals and parasites.** Chickens were housed at Gore Hill Research Laboratories at 21°C with a 12-h light-dark cycle and with free access to food and water. The Houghton strain of *E. maxima* was originally provided by Martin Shirley (Institute for Animal Health, Compton, Newbury, Berkshire, United Kingdom). Oocysts were passaged through 4-week-old chickens (Australorps) and purified as described previously (29). Oocysts were then resuspended at 2.5 × 10⁵/ml in 2% potassium dichromate at 4°C until required. Gametes were isolated at 134 h postinfection at the Swedish University of Agricultural Sciences (Uppsala, Sweden), purified from infected chicken intestines by techniques published previously (36), and stored as aliquots at −80°C until required. For an example of the purity of the gamete preparation, see Fig. 3D.

**Antibodies.** Development of the mouse anti-gam56 monoclonal antibody (MAB) 1E11-11 has been described previously (35). Antiserum (serum P) against affinity-purified gametocyte antigen (APGA) was developed in chickens against good culture system for studying the sexual stages of *Eimeria* and problems associated with isolating large quantities of parasites. However, with the advent of new technologies in proteomics and molecular biology, together with improved methods for the increased production of the sexual stages of development, we have now isolated and sequenced five oocyst wall proteins and have shown that four of them are derived from two larger, tyrosine-rich precursor proteins found in macrogametes. In addition, we provide evidence to support the hypothesis that the oocyst wall of *E. maxima* is stabilized through the cross-linking of tyrosine residues in such proteins.
an enriched fraction of the native forms of gam56 and gam82 (35). Mouse anti-recombinant gam56 (anti-r56) polyonal antibodies (serum 2562) were generated as follows. Mice were immunized three times, at 3-week intervals, with 5 µg of purified r56. Blood was collected after the second immunization in Vacutainer tubes (BD), and serum was harvested according to the manufacturer’s instructions. r56 was generated as follows. The forward primer SB74 (5’-CGCGGATCCGGACTCTGTTGGAAGACATGCT-3’) (= G + T; Y = C + T) and reverse primer SB75 (5’-GGCAATCTGGGGATCAGGTCGGAGATGGA3’) were used at 10 µM each to amplify, by PCR, the region corresponding to bp 172 to 1137 (GenBank accession number AJ129951) of the coding region (C) of gam56. cDNA, synthesized as described previously (7), was used as template. cDNAs, together with the primers, were heat denatured at 70°C for 5 min and kept on ice prior to amplifications. The Advantage 2 PCR enzyme system (Clontech) was used in the amplifications under the following conditions: initial denaturation (94°C, 3 min) for one cycle and denaturation (94°C, 30 s), annealing (70°C, 30 s), and extension (72°C, 3 min) for 30 cycles. PCR products were then purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions prior to digestion with BamHI and EcoRI. The digested purified PCR products were cloned and inserted into the vector TrcHis B (Invitrogen) according to the manufacturer’s instructions. Expressed proteins were purified from bacterial lysates by nickel-nitrilotriacetic acid chromatography (Qiagen) as described in the manufacturer’s instructions and then lyophilized and stored at −20°C. Proteins were reconstituted in water when required.

Isolation of purified oocyst wall fragments. Purified oocyst wall fragments were prepared as described previously (14) and stored as pellets at −80°C until required. Briefly, sporulated oocysts (~106) were washed in distilled water (1,750 × g, 10 min, 4°C) to remove the 2% potassium dichromate within which they were stored. Oocysts were then resuspended in 40 mM Tris-Cl (pH 9) and sonicated (Cole Parmer high-intensity ultrasonic processor [2-mm microtip; output setting, 50 W]) for 10-s intervals over 1 min. The sonicate was then centrifuged (2,500 × g, 10 min, 4°C) in a swing-out rotor. When the supernatant was clear, it was discarded, and the oocyst-sporocyst wall pellet was stored at −80°C until required.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie blue staining of proteins, and immunoblotting were carried out essentially as described previously (5). Briefly, intact gametocytes were resuspended in Laemmli sample buffer (21) in the presence of 5% β-mercaptoethanol, heated for 2 min at 100°C, and centrifuged (12,000 × g, 2 min) to remove insoluble proteins and aggregated matter prior to SDS-PAGE (21) on a 4 to 20% polyacrylamide gel (GelBond). Oocysts were washed in distilled water (1,750 × g, 10 min, 4°C) to remove the 2% potassium dichromate within which they were stored. They were then resuspended in 40 mM Tris-Cl (pH 9) and mechanically disrupted by being vortexed in the presence of glass beads prior to the addition of sample buffer. Purified oocyst wall fragments (~106 parasite equivalents) were separated by using the SDS-PAGE Tris-Tricine system (27). Membranes were probed after transfer with the following sera: anti-r56 antisera (serum 2562; 15,000) anti-APGA antiserum (serum P: 1:1,000), normal mouse serum, or normal chicken serum.

N-terminal sequencing. Purified oocyst wall fragments (~106 parasite equivalents) were separated on a 4 to 20% polyacrylamide gel by using the SDS-PAGE Tris-Tricine system (27), transferred to a polyvinylidene difluoride membrane, and stained with Coomassie blue, and the bands were isolated as described previously (5). Unsporulated oocysts (~106) were disrupted mechanically as described above, and the samples were processed and separated by two-dimensional gel electrophoresis as described previously (5). N-terminal sequences were determined by Edman degradation at the Australian Proteome Analysis Facility (Sydney, Australia).

Microscopy. Purified sporulated oocysts were washed with distilled water (1,750 × g, 10 min) to remove the 2% potassium dichromate within which they were stored. Gametocytes that had been stored at −80°C were used. An Olympus BX51 reflected fluorescence microscope with a PM-30 automatic photomicrographic system was used. A drop of purified oocysts, gametocytes, or purified oocyst wall fragments was placed under a coverslip on a glass slide, and autofluorescence was visualized by using a narrow-band 330- to 385-nm filter. Indirect staining of oocysts with the MAB anti-gam56 (1E11-11) followed by a peroxidase anti-mouse immunoglobulin F(ab’2)-fluorescein isothiocyanate conjugate has been described elsewhere (7). Parasites were viewed under bright-field microscopy or with a narrow-band 470- to 490-nm filter for immunofluorescence detection. Infections of chickens with *E. maxima* (Houghton strain) oocysts and the preparation of gut tissue sections taken at 164 h postinfection have been described elsewhere (7). Either sections were pretreated for 15 min at 37°C with 3% hydrogen peroxide and washed in water prior to immunostaining or left untreated. Peroxidase activity was measured by incubating the sections with a peroxidase substrate consisting of 0.5 mg of 3,3-diaminobenzidine tetrahydrochloride per ml, 0.2 mg of cobalt chloride per ml, and 0.3 mg of urea hydrogen peroxide per ml in 0.05 M Tris and 0.15 M sodium chloride (Sigma FAST). They were left for 15 min at 37°C and then washed with water before bright-field microscopy.

Measurement of DOPA and dihydroxy. *E. maxima* oocysts were isolated, purified, and left at 25°C to sporulate as described above. Sporulated oocysts were then cleaned in hypochlorite solution and stored in 2% potassium dichromate at 4°C until required. Oocysts were isolated by centrifugation (1,750 × g, 10 min, 22°C) and washed twice in 13 ml of sterile distilled water to remove any residual dichromate. The pelleted oocysts (~6 × 108 to 7.5 × 106 total per sample) were then resuspended in 100 µl of 40 mM Tris-Cl (pH 9.5). Glass beads (710-1,180 µm; ~5 g; prewashed in 40 mM Tris-Cl [pH 9.5]) were added to the isolated oocysts, and each sample was vigorously vortexed for 7 min (until >90% of the oocysts had been broken). Solutions containing the disrupted oocysts were recovered from the glass beads by washing the beads with another 500 µl of 40 mM Tris-Cl (pH 9.5). The collected samples (~600 µl) were then transferred to 1-ml brown glass autosampler vials (Alltech) treated with 1 mg of sodium boro-hydrate per ml, 0.03% sodium deoxycholate, and 50% (wt/vol) trichloroacetic acid. Samples were centrifuged at 4,000 × g for 2 min, and the protein pellets were washed twice with cold acetone and freeze-dried. The vials were then placed in a Picotag reaction vessel (Alltech) containing 1 ml of 6 M HCl and 50 µl of 1M hydrochloric acid. The reaction vessels were evacuated and then incubated at 110°C for 16 h. These hydrolysates were subsequently freeze-dried and redissolved in water (200 µl) for amino acid analysis. Samples were then separated by high-pressure liquid chromatography (HPLC) on a Zorbax octadecyl silane column (250 by 4.6 mm; 5 µm diameter) with a flow rate of 1 ml/min. Dihydroxy and 3,4-dihydroxyphenylalanine (DOPA) were eluted by using the following gradient of solvent A (100 mM sodium perchlorate in 10 mM sodium phosphate [pH 2.5]) and solvent B (80% [vol/vol] methanol in water): isocratic elution with 0% solvent B for 23 min, then to 6% solvent B in 9 min, further elution at 6% solvent B for 11 min before changing to 50% solvent B in 1 min, isocratic elution at 50% solvent B for 10 min before changing back to 0% solvent B over 5 min, and then a reequilibration with 100% solvent A for 13 min. Eluted materials were detected by serial UV-visible and fluorescence detectors. Tyrosine levels were quantified by UV absorbance, whereas DOPA and dihydroxy levels were quantified by fluorescence with a λ<sub>ex</sub> of 280 nm and a λ<sub>em</sub> of 320 nm for DOPA and λ<sub>ex</sub> of 280 nm and a λ<sub>em</sub> of 410 nm for dihydroxy. Quantification was achieved by comparison of peak areas with appropriate standard curves constructed with authentic materials. The standards were used periodically during each HPLC run. Every HPLC run was performed after the system (column, detectors, and lines) had been fully equilibrated with the fresh batch of buffer made for that run. Each HPLC injection (i.e., sample and/or standard) was performed when the trace was returned to a baseline level. The machine automatically zeroed the baseline trace at the 0-min time point for each injection. This method of determining the levels of dihydroxy and DOPA after HCl-mercaptoacetic acid hydrolysis of oocysts has been examined previously for the artificial formation of oxidized tyrosine during the process. With authentic tyrosine and other proteins, only very low levels of these products were detected (15).

RESULTS

The gametocyte glycoproteins gam56 and gam82 are proteolytically processed into smaller peptides that are incorporated into the developing oocyst wall of *E. maxima*. We have shown previously that a MAB (1E11-11) to the gametocyte glycoprotein gam56 is capable of providing partial passive immunity to coccidiosis in chickens and localizes to the wall-forming bodies in macrogametes and to the oocyst wall in *E. maxima* (7, 35). These observations indicated that some oocyst wall proteins and gam56 share a common epitope and might therefore arise from the same precursor protein. An investigation was carried out to address this possibility by probing immunoblots of ga-
metocyte and oocyst extracts with a polyclonal antiserum that recognizes gam56. Analysis by SDS-PAGE and immunoblotting revealed that antibodies raised to a recombinant version of gam56 (anti-r56) recognized the native form of gam56 (apparent molecular mass, 56 kDa) in purified gametocyte extracts (Fig. 1A), as expected. In addition, these antibodies recognized a ~33-kDa protein (designated wp33) in unsporulated oocyst extracts (Fig. 1A), in sporulated oocyst extracts (Fig. 1A), and in purified wall extracts (Fig. 1B). The anti-APGA antiserum, raised to an enriched fraction of the native forms of gam56 and another E. maxima gametocyte antigen, gam82 (6), also recognized a ~33-kDa oocyst wall protein (Fig. 1B). This protein was more abundant in unsporulated oocyst preparations than in sporulated oocyst preparations (Fig. 1A), where identical parasite equivalents ($5 \times 10^5$) were loaded on the gel, and purified oocyst wall preparations, where $10^6$ parasite equivalents were loaded on the gel to achieve a strong signal. The observation that the level of detection of wp33 decreased during sporulation and wall formation might be representative of the translocation of the soluble form of this protein to the wall. However, it cannot be excluded that the level of detection of these proteins might reflect the ability of the antisera to recognize different conformations of the wall proteins during development. A 56-kDa protein, probably representing gam56, was detected in unsporulated and sporulated oocyst extracts only when protein loadings on polyacrylamide gels were increased fourfold (data not shown). A predominant band at ~21 kDa was also observed in lanes containing gametocyte extracts; the origin of this protein remains unknown (Fig. 1A). When membranes were probed with normal mouse serum (C2) (Fig. 1) or normal chicken serum (C1) (Fig. 1B), no protein bands were detected.

Recognition of both a 56-kDa gametocyte glycoprotein (gam56) and a ~33-kDa oocyst protein (wp33) with the anti-r56 antibody, and the detection of residual gam56 in oocyst extracts, provided biochemical evidence indicating that gam56 and wp33 share a common epitope. Since the gene encoding gam56 has been cloned and sequenced (7), the relationship between gam56 and wp33 was analyzed further at the amino acid level. Proteins in an unsporulated oocyst extract were separated by two-dimensional gel electrophoresis and transferred to a membrane, and the spot corresponding to wp33 was isolated. The N terminus of this protein was sequenced and mapped to amino acids 21 to 35 in the gam56 protein, represented schematically in Fig. 2B. Together with the observation that gam56 is encoded by a single RNA transcript (7), and with the biochemical data presented in Fig. 1, these findings provided evidence supporting the hypothesis that gam56 is a precursor protein that is proteolytically processed during development to give rise to a ~33-kDa (wp33) oocyst wall protein in Eimeria.

The anti-r56 antibody used here was generated against amino acids 24 to 346 of gam56 and not the entire coding region. This antibody, as well as anti-APGA, an antibody that was raised against an enriched fraction of native gametocyte antigens predominating in gam56 and gam82, recognized only a ~33-kDa (wp33) protein in oocyst wall preparations; they did not recognize any smaller proteins (10 to 14 kDa) reported previously to be the major components of the oocyst wall in Eimeria (14, 20, 31). Thus, an investigation was carried out to elucidate the relationship between these smaller 10- to 14-kDa oocyst wall proteins and gam56 in E. maxima.

Oocyst wall extracts purified from E. maxima were analyzed by SDS-PAGE (Fig. 2A). Two clusters of proteins were detected by Coomassie blue staining, one at 8 to 12 kDa (designated wp8, wp10, and wp12) and another at 29 kDa (designated wp29). The 8- to 12-kDa cluster was not detectable by silver staining (data not shown). Each protein in the 8- to 12-kDa

![FIG. 1. Developmental processing of the gametocyte 56-kDa glycoprotein, gam56, to a ~33-kDa oocyst wall protein in E. maxima. (A) Immunoblot of extracts from gametocytes (gam), unsporulated oocysts (u-oocyst), and sporulated oocysts (sp-oocyst) probed with mouse anti-r56 antibody or control normal mouse serum (C2). Gels were loaded with $5 \times 10^5$ parasite equivalents for all stages. (B) Immunoblot of purified oocyst wall fragments probed with chicken anti-APGA antibody, control chicken serum (C1), mouse anti-r56 antibody, or control normal mouse serum (C2).](image-url)
cluster was isolated from purified oocyst wall extracts by SDS-PAGE and transferred to a membrane, and the N termini were sequenced. The sequence corresponding to the N terminus of wp29 (Fig. 2A) was unrelated to gam56 (5, 7) or to another E. maxima gametocyte antigen, gam82 (5, 6), and showed no similarity to any other previously published proteins as revealed by BLAST (1) searches of all DNA and protein databases. Since this sequence was not found in gam56, this also showed that wp29 and wp33 were unrelated. In contrast, the sequence corresponding to the N terminus of wp12 was identical to the tyrosine-rich domain I of gam56 (Fig. 2B), mapping to amino acids 240 to 254 of gam56 (7). In addition, N-terminal sequences corresponding to wp8 and wp10 were identical to tyrosine-rich domains I (amino acids 282 to 296) and II (amino acids 418 to 431), respectively, of gam82.

**Oocysts autofluoresce, which is indicative of the presence of dityrosine cross-links.** Since the gametocyte and oocyst proteins were shown to be rich in the amino acid tyrosine, their role in wall hardening was investigated in E. maxima. Tyrosine-derived oxidation products, and particularly the dimeric material dityrosine, which can cross-link proteins, have been implicated in wall hardening and cuticle formation in a number of organisms, including parasites, yeasts, insects, worms, and sea urchins (8, 9, 16, 19, 32), and dityrosine can be detected easily.
by its characteristic UV fluorescence or by amino acid analysis of hydrolysates (2, 8, 12).

When *E. maxima* parasites were examined microscopically with an excitation wavelength of 330 to 385 nm, only oocysts (Fig. 3A) and developing oocysts (Fig. 3D) autofluoresced blue. Macrogametes did not autofluoresce (Fig. 3D). Purified oocyst wall fragments also fluoresced (Fig. 3E), as did sporocyst walls (Fig. 3E). The pattern of autofluorescence colocalized clearly with anti-gam56 MAb (1E11-11) binding to the oocyst wall (Fig. 3B). Not all parasites were stained with the antibody, and not all oocysts autofluoresced (compare Fig. 3A and B to C). This might be a reflection of the plane of view at which the oocysts were examined, or alternatively, it might represent a population of aging oocysts in which the dityrosine bonds were deteriorating. If the latter was true, then the fact that the oocysts still appeared intact microscopically indicates that dityrosines are not the sole structural component contributing to the integrity of the wall.

**Dityrosine and DOPA are biochemically detectable in oocyst extracts.** The possibility that the oocyst wall was hardened by mechanisms other than dityrosine cross-link formation was investigated. Dityrosine and DOPA, another product of tyrosine oxidation implicated in the synthesis of “extraorganismic” structural materials (4, 32, 33), were identified and quantified in hydrolysates of oocysts by comparison of retention times of the putative peaks with those of authentic materials and their characteristic UV and fluorescence spectra (Fig. 4). Both DOPA (7.2 ± 2.4 mmol/mol of tyrosine) and dityrosine (3.4 ± 1.1 mmol/mol of tyrosine) were readily detectable, at high concentrations, in amino acid hydrolysates from *E. maxima* oocysts, supporting a role for tyrosine oxidation products in oocyst wall formation in the parasite *E. maxima*.

**Peroxidase activity is detectable in the wall-forming bodies of macrogametes.** An investigation was carried out to identify other components of the molecular machinery underlying di-
tyrosine and DOPA formation and oocyst wall hardening in *E. maxima*, with particular emphasis on peroxidase enzymes. The role of peroxidases in catalyzing dityrosine cross-linking in vivo is well established in mammalian systems (17, 18). Peroxidases have also been identified in the sea urchin, and these have been implicated in the hardening of the fertilization membrane (16). In addition, in vitro cross-linking of dityrosine proteins by horseradish peroxidase has been demonstrated for silk fibroin (3) and for CUT-2, a protein component of the cuticle of the nematode *Caenorhabditis elegans* (25).

Chickens were infected with *E. maxima* oocysts, and intestines were removed at 164 h postinfection. Histological tissue sections were prepared, and peroxidase activity was measured by incubating sections in the peroxidase substrate dianinobenzidine in the presence of hydrogen peroxide. Only the wall-forming bodies of microgametes stained clearly with the substrate, as seen microscopically (Fig. 5A and B). Microgametes were negative for peroxidase activity (Fig. 5B). When sections were preincubated with 3% hydrogen peroxide prior to the addition of substrate, the activity was reduced (Fig. 5C) compared to that in untreated sections (Fig. 5D) in the same experiment, and when sections were pretreated with 50 mM dithiothreitol, the activity was abolished.

**DISCUSSION**

The oocyst wall of *Eimeria* is composed of tyrosine-rich proteins. The work presented here has shown that the oocyst wall of *E. maxima* is composed of at least two clusters of proteins, 8 to 12 kDa and 29 to 33 kDa in size, present at various levels in the wall, some of which are derived from the gametocyte glycoproteins gam56 and gam82. Both wp33 and wp12 are derived from gam56, whereas wp8 and wp10 are derived from gam82. Since sequences corresponding to the amino termini of these proteins have been determined, the predicted cleavage sites for their generation can also be calculated. Their sizes would therefore suggest that the gam56 and gam82 proteins undergo both N-terminal and C-terminal cleavage to generate these fragments. Although a ~30-kDa protein and up to two ~14-kDa wall proteins have been described previously for *Eimeria acervulina*, *Eimeria tenella*, and *E. maxima* (14), Eschenbacher et al. (14) did not achieve the level of resolution of the proteins seen here; this was achieved only by replacing the routinely used SDS-PAGE Tris-glycine system for separation with a Tris-Tricine system.

Amino acid sequencing of oocyst wall proteins from *E. maxima* and immunoblotting of gametocyte extracts and purified oocyst wall fragments with antibodies that recognize the gametocyte glycoproteins gam56 and gam82 were used here, and previously (6), to determine the relationship between gametocyte and oocyst wall proteins. The anti-r56 antiserum used here was raised against amino acids 24 to 342 of gam56, a region that includes sequences that encode the N terminus of the mature protein and tyrosine-rich domain I but excludes most of the C-terminal proline-rich region. Since this antiserum did not recognize the proteins in the 8- to 12-kDa cluster of oocyst wall proteins, it is possible to conclude that the r56 fragment, used to generate this antiserum did not contain the epitope that would allow the antibody to recognize the 8- to 12-kDa oocyst wall proteins. In other words, the tyrosine-rich domain I alone is not antigenic. Recent studies supporting this hypothesis have shown that both the anti-r56 and anti-APGA antibodies did not recognize bacterially expressed recombinant proteins generated from the tyrosine-rich domains of gam56 and gam82 by immunoblotting (K. Mai, S. Belli, M. Wallach, N. Smith, unpublished data). However, antibodies to an enriched preparation of the native forms of gam56 and gam82 (anti-APGA) and an antibody raised to the recombinant form of the entire coding region of gam82 (anti-r82) (6) did not recognize the 8- to 12-kDa cluster of oocyst wall proteins either. The fact that the anti-APGA serum did not recognize the 8- to 12-kDa cluster was unexpected. Since Eschenbacher et al. (14) have successfully raised antibodies to a 14-kDa oocyst wall protein from *E. acervulina* and *E. tenella* in rats, the
Findings presented here suggest that the epitope recognized by the antibodies, anti-APGA and anti-r82, in the precursor proteins is not maintained after processing; that is, the epitope might be conformation sensitive. In line with this, the MAb, 1E11-11, raised to gam56 is epitope conformation sensitive and more readily recognizes the oocyst wall of purified, intact oocysts by immunofluorescence and not by immunoblotting. It is therefore difficult to definitively conclude which oocyst wall protein MAb 1E11-11 recognizes. Generation of antibodies to the purified oocyst wall proteins might help elucidate which epitopes are recognized by all of these antibodies.

Although immunoblotting provided some clue as to the relationship between the gametocyte antigens and the oocyst wall proteins, their relationship was further elucidated through the determination of the amino acid sequences corresponding to the N termini of the oocyst wall proteins. It was clear that the 8- and 10-kDa proteins were derived from gam82 and that the 12-kDa protein was derived from gam56, even though antibodies raised to recombinant and native forms of the gametocyte proteins did not recognize the oocyst wall proteins by immunoblotting. All the antibodies, i.e., anti-APGA, anti-r56, and anti-r82 (6), did, however, recognize a ~30-kDa protein in the oocyst wall extracts by immunoblotting. This would suggest that this protein is more antigenic than the smaller tyrosine-rich proteins or that the epitope is maintained during processing. In addition, the data presented here indicated that the ~30-kDa band seen on Coomassie blue-stained polyacrylamide gels represented a mixture of different proteins with similar masses: one related to gam56 (wp33), one related to gam82 (6), and the other of unknown origin (wp29). These

**FIG. 5.** Peroxidase activity in the wall-forming bodies of macrogametes of *E. maxima*. (A and B) Intestinal tissue sections taken at 164 h postinfection with *E. maxima* and stained with diaminobenzidine in the presence of hydrogen peroxide. Magnifications, ×200 (A) and ×400 (B). (C and D) Intestinal tissue sections taken at 164 h postinfection and stained with diaminobenzidine with and without pretreatment with 3% hydrogen peroxide. Magnification, ×400. WFB, wall-forming body.
findings suggest that the parasite undergoes extensive processing of a number of preexisting proteins in the development of the oocyst wall.

The oocyst wall is a robust structure that is resistant to a number of common protein extraction procedures. Therefore, it cannot be excluded that the levels of detection of these proteins in the oocyst wall might reflect their overall extractability from this structure. Mild conditions were deliberately used here to extract proteins from the oocyst wall in an effort to maintain the antigenicity of the proteins. However, under such conditions, it has been estimated that less than 10% of the oocyst wall is solubilized (31), and therefore, the proteins described here might represent only a few of the total number of proteins involved in wall formation.

Although proteins wp8, wp10, and wp12 did not share any sequence homology to the N terminus of the ~14-kDa wall protein in E. tenella and E. acervulina (14), they did share one common feature: all proteins were rich in the amino acid tyrosine. The gametocyte glycoproteins gam56 (7) and gam82 (6) in E. maxima have also been shown previously to be rich in tyrosine. In addition, tyrosine-rich proteins have also been identified as the major component of egg shells in the blood fluke of humans, Schistosoma mansoni (9), and the liver fluke, Fasciola hepatica (32), as well as in the byssus (attachment foot) of the Zebra mussel, Dreissena polymorpha (4). Together, these findings suggest that tyrosine-rich proteins are important constituents of extracellular matrices in a number of organisms, including Eimeria.

The oocyst wall is composed of cross-linked protein. SDS-PAGE and immunoblotting with anti-gam56 antibodies did not reveal the presence of any high-molecular-weight, oligomeric forms of the oocyst wall proteins or any cross-linked intermediates. It is possible that the antibodies used in these experiments did not recognize the processed, cross-linked wall proteins by immunoblotting. Alternatively, as seen in in vitro cross-linking experiments with cuticulin, the major component of the nematode C. elegans (25), the oocyst wall proteins might form large insoluble aggregates that are not detected by SDS-PAGE and immunoblotting.

The autofluorescence properties of Eimeria have been exploited by others and used diagnostically to identify species of Eimeria oocysts in swine feces (10) and fish tissues (11), without prior knowledge of the molecular basis of this fluorescence. Here, we have provided evidence for the natural autofluorescence of Eimeria oocysts being due to the presence of dityrosine cross-links. This cross-linking has been measured biochemically by HPLC and shown to be present in the oocysts at very high levels (3.4 mmol/mol of tyrosine); the level of this material is much higher than that detected in many other physiological and pathological samples (12), consistent with this material being generated in a deliberate, enzyme-mediated reaction rather than as a result of the exposure of the oocysts to oxidative stress. The detection of very high levels of DOPA, in addition to dityrosine, in oocyst extracts indicates that dityrosine-mediated cross-linking of proteins may not be the sole mechanism of wall polymerization; DOPA-mediated cross-links may also be a major feature. As DOPA is not a common constituent of proteins, there is reason to believe that a mechanism that involves “quinone tanning” might also be an important component of oocyst wall formation in Eimeria (22, 33).

In a review by Waite (33) it was pointed out that quinone-tanned structures involving DOPA-containing proteins have been shown to be poorly antigenic and dissolved by sodium hypochlorite. In Eimeria, oocysts are routinely washed in a 10% sodium hypochlorite solution as the final step in their purification (29) and, when examined microscopically, remain unaltered. Although attempts to assess the antigenicity of the oocyst wall in Eimeria have not been reported, antibodies to gametocyte glycoproteins have been shown to recognize the oocyst wall in E. maxima by immunofluorescence staining (Fig. 3B), indirectly providing evidence that the oocyst wall contains antigenic determinants. In addition, although oxidized DOPA can also emit blue fluorescence, it does not contribute significantly to the autofluorescence seen here, which is typical of oxidized tyrosine- and o-o’-coupled dimers in the excitation range of 330 to 385 nm (30). Therefore, the evidence presented here supports the hypothesis that both dityrosine- and DOPA-derived cross-links are major components of the oocyst wall in Eimeria. The combined presence of DOPA- and dityrosine-containing proteins in the oocyst wall might have a complementary effect, conferring a balance between rigidity and flexibility in the wall. The absence of one or the other component would therefore compromise the viability of the oocyst and, consequently, parasite survival. The role of DOPA (and its oxidation products) in wall formation in Eimeria is under further investigation.

Peroxidase enzymes have been implicated in the formation of dityrosine cross-links, and peroxidase activity was detected in the wall-forming bodies of macrogametes. The observation that peroxidase activity was not detected in microgametes in the same section of infected intestine analyzed indicated that the activity measured was specific. However, it cannot be excluded that other enzymes implicated in the quinone tanning of DOPA-containing proteins, such as polyphenol oxidase (32), are not present in these parasites.

Concluding remarks. These findings, together with previous investigations describing oocyst wall formation in Eimeria (5–7, 14, 31), provide evidence that the molecular machinery involved in the assembly of the oocyst wall, including precursor proteins, processing enzymes, cross-linking enzymes, and cofactors, are housed in the wall-forming bodies in macrogametes. To date, the signals that trigger the aggregation and fusion of the wall-forming bodies and their discharge to form the oocyst wall remain elusive; however, they are thought to occur during the fertilization of macrogametes with microgametes (28). The parasite has thus developed a resourceful mechanism by which it cleaves previously synthesized proteins to form the oocyst wall, a process that would be more time efficient than transcribing proteins from genes de novo. Such a mechanism would enable the quick encapsulation of the fragile parasite, protecting it from exposure to adversity within the lumen of the gut and the exterior environment of the host prior to excretion.

The work presented here provides further insights into the molecular basis of oocyst wall development in Eimeria. It brings together ~50 years of research in the field, where the suggestion that the oocyst wall of Eimeria was composed of quinone-tanned protein was first put forward by Monné and Hö nig in 1954 (24) and where Eschenbacher et al. (14) speculated that the ~14-kDa protein was incorporated into the oocyst wall through a tyrosine cross-linking mechanism called
tanning. *E. maxima* thus provides an attractive model to investigate oocyst wall formation in other cyst-forming coccidia for two reasons: (i) the oocysts are large (30 µm in diameter [23]) and easy to visualize microscopically, and (ii) the purification of large quantities of macrogametes and oocyst stages is feasible. In other coccidia, such as *Plasmodium*, *Toxoplasma*, *Cyclospora*, and *Neospora*, the isolation of gamete and oocyst stages in sufficient quantities to analyze is sometimes impossible. Therefore, any discoveries elucidating the molecular basis of oocyst wall formation in *E. maxima* may identify novel targets for the control of malaria, toxoplasmosis, cyclosporosis, and neosporosis, diseases that have a strong impact on economies and health worldwide.

ACKNOWLEDGMENTS

This study was financed by the Commonwealth Government of Australia, Department of Education, Training and Youth Affairs, in partnership with ABIC Veterinary Products Ltd., Israel, through an Australian Research Council SPIRT grant to Nicholas Smith and Michael Wallach and an ARC fellowship to Michael Davies. The provision of HPLC facilities by the Wellcome Trust is also gratefully acknowledged. We are grateful to Martin Shirley (Institute for Animal Health, Compton, United Kingdom) for providing the Houghton strain of *E. maxima* and to David Witcombe (Institute for the Biotechnology of Infectious Diseases, Sydney, Australia) and Per Thebo (Swedish National Veterinary Institute, Upsala, Sweden) for assistance in the isolation of gametocytes and oocysts. We are also grateful to J. Herbert Waite (University of California, Santa Barbara) for his critical assessment of the work presented here.

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