Aminopeptidase N1 (EtAPN1), an M1 Metalloprotease of the Apicomplexan Parasite *Eimeria tenella*, Participates in Parasite Development

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Aminopeptidases N are metalloproteases of the M1 family that have been reported in numerous apicomplexan parasites, including *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, and *Eimeria*. While investigating the potency of aminopeptidases as therapeutic targets against coccidiosis, one of the most important avian diseases caused by the genus *Eimeria*, we identified and characterized *Eimeria tenella* aminopeptidase N1 (EtAPN1). Its inhibition by bestatin and amastatin, as well as its reactivation by divalent ions, is typical of zinc-dependent metalloproteases. EtAPN1 shared a similar sequence, three-dimensional structure, and substrate specificity and similar kinetic parameters with A-M1 from *Plasmodium falciparum* (PfA-M1), a validated target in the treatment of malaria. EtAPN1 is synthesized as a 120-kDa precursor and cleaved into 96-, 68-, and 38-kDa forms during sporulation. Further, immunolocalization assays revealed that, similar to PfA-M1, EtAPN1 is present during the intracellular life cycle stages in both the parasite cytoplasm and the parasite nucleus. The present results support the hypothesis of a conserved role between the two aminopeptidases, and we suggest that EtAPN1 might be a valuable target for anticoccidiosis drugs.

Coccidiosis is the most important avian disease, being responsible for major economic losses in the poultry industry (1, 2). It is caused by intestinal infection of chickens with parasites of *Eimeria* spp. The life cycle of *Eimeria* is divided into an intestinal stage and an environmental stage. The intestinal stage involves the invasion of epithelial cells of the chicken intestine by sporozoites, differentiation into schizonts, and replication of merozoites within epithelial cells, followed by production of male and female gametes, fertilization, and formation of unsporulated oocysts. The environmental stage involves the release of unsporulated oocysts and their maturation, or sporulation, into infectious sporulated oocysts (3). These infectious diseases are currently controlled by the preventative addition of anticoccidial drugs to poultry feed or by administration of live vaccines (4). However, the increase of drug-resistant parasite populations and the cost of live vaccines underline the need to find alternative targets and drugs.

The *Eimeria* genus belongs to the apicomplexa phylum, a group of medically and economically important parasites including *Plasmodium* spp. and *Toxoplasma gondii*, which cause the human diseases malaria and toxoplasmosis, respectively. Within the seven species of *Eimeria* that infect poultry, *Eimeria tenella* is one of the most virulent (5), and its genome has been sequenced and partially annotated (http://www.genedb.org/Homepage/Etenella). Two cellular models are usually used for in vitro studies of intracellular parasite development: the MDBK cell line and primary chicken kidney cells (PCKCs) (6, 7). It has been hypothesized that proteases play crucial functions in the life cycle of *E. tenella*, as observed for other apicomplexa. Previous studies have supported this hypothesis (8). Among parasitic proteases, metalloproteases have been related to pathogenesis and are involved in processes such as immunity evasion, development, and metabolism (9–11). Analysis of the *E. tenella* genome revealed the presence of at least 45 proteases, 31% of which were metalloproteases, that are transcribed in different stages of the parasite life cycle (12).

The presence of an active metalloprotease of the M1 family (aminopeptidase M1, alanyl aminopeptidase, aminopeptidase N) has been reported in *E. tenella* oocyst lysates throughout sporulation (13). Recent analysis of the genome identified two putative aminopeptidase N-like proteases that belong to the M1 metalloprotease family (*E. tenella* aminopeptidase N protease 1 [EtAPN1] and EtAPN2) (12). To date, no data are available on the implication of *E. tenella* aminopeptidase N in intracellular stages, except for the detection of a peptidase activity against homoarginine-pepetyl-7-amino-4-methyl coumarin (H-Arg-AMC) in merozoite lysates (13). In contrast, the aminopeptidase N of *Plasmodium falciparum*, A-M1 (PfA-M1), is well characterized. PfA-M1 is found in various locations, such as the parasitophorous vacuole, food vacuole (14, 15), cytoplasm (15, 16), and nucleus (17). Bestatin-based studies revealed that PfA-M1 is involved in parasite metabolism and participates in the last steps of hemoglobin degradation. Moreover, PfA-M1 might be involved in other biological processes, since it is also found in the nucleus of trophozoites, an unrelated organelle for hemoglobin degradation (10, 14, 17). We...
examined the presence of the active aminopeptidase of *E. tenella* in both the development and sporulation phases of the parasite life cycle using bestatin and specific aminopeptidase fluorescent substrates. Herein, we specifically focused on EtAPN1 and investigated its biochemical and molecular properties. We produced a functionally active recombinant EtAPN1 (EtAPN1r), characterized its main enzymatic properties, and compared them with those of PfAP-M1. In addition, we studied the pattern of expression of EtAPN1 during *E. tenella* sporulation and its subcellular localization during the development of the parasite in intracellular stages from sporozoites to gametes. To our knowledge, this is the first report showing that EtAPN1 is localized into the cell nucleus during infection. This novel result is important in light of the control of coccidiosis. In addition, our bestatin assays suggested that EtAPN1 may be a valuable candidate for anticoccidial chemotherapy. More specific inhibitors are needed for proper understanding of the potential of EtAPN1 as a drug target.

**MATERIALS AND METHODS**

**Ethics statements.** Experimental protocols were designed in compliance with French law (Décret 2001-464, 29 May 2001) concerning the use of laboratory animals. Care and euthanasia of animals were practiced according to national ethical guidelines and approved by the Ethics Committee of the Région Centre (CL2007-36). The authors are committed to the principles of the 3Rs: reduction, refinement, and replacement of experimental animals.

**Parasite harvest.** Groups of outbred PA12 chickens (age, 4 to 6 weeks) were infected orally with 10⁴ and 10⁵ sporulated oocysts of the *E. tenella* Wis, Wis yellow fluorescent protein-positive (YFP⁺), and Wis96 (18) strains, respectively. The Wis YFP⁺ strain was obtained by P. Brossier: Wis parasites were transfected with a plasmid carrying the YFP gene under the control of the *E. tenella* mic1 promoter. Unsporulated oocysts were harvested from infected ceca 7 or 5 days postinoculation for the Wis and Wis96 strains, respectively. Unsporulated oocysts were purified using sodium hypochlorite and MgSO₄ as described previously (19). For the sporulation time course studies, oocysts were suspended in water containing 2% (wt/vol) potassium dichromate and incubated for various times (0, 6, 12, 24, 48, and 72 h) at 26°C. Sporozoites were obtained from fresh fully sporulated oocysts, and after breaking the oocyst walls with glass beads, sporozoites were incubated in the excystation medium (0.25% [wt/vol] trypsin and 0.5% [wt/vol] biliary salts in 10 mM phosphate-buffered saline [PBS], pH 7.4) at 41°C for 1 h. Sporozoites were purified by a two-step filtration protocol, first on cotton and then on polycarbonate filters (pore size, 5 μm; Whatman). Second-generation merozoites were recovered from chicken cecal mucosa 112 h after infection with 5 × 10⁵ sporulated oocysts, as previously described (20).

**Bestatin effect on cell culture.** Freshly excysted, purified Wis YFP⁺ sporozoites (2 × 10⁵) were incubated with 2 × 10⁴ MDBK cells in 24-well plates in the presence of bestatin (50 to 500 μM) for 4 h and 96 h at 41°C in 5% CO₂, as previously described (21). The absence of cytotoxicity induced by bestatin was verified using an (3,45-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (22). Infected cells were washed at 4 h postinfection (p.i.) (for the invasion assay) or at 96 h p.i. (for the development assay) and fixed in 2.7% paraformaldehyde (PFA) for immunofluorescence assay. Monolayers were mounted in Vectashield mounting medium containing 1.5 μg/ml of DAPI (4’,6-diamidino-2-phenylindole; Clinisciences, France) to label the nuclei. Infected cells were examined by fluorescence microscopy (Zeiss Axiosvert 200 microscope; Carl Zeiss, Germany). At least 200 cells were counted for each condition, with two wells per condition, in three independent experiments. At 72 h p.i., the percentage of schizonts was calculated as follows for the development assay: number of schizonts × 100/(number of sporozoites + number of schizonts). At 96 h p.i., the merozoites present in the supernatant were counted. Values are reported as means ± standard errors.

**Enzymatic activity assays.** Oocyst lysates (10⁴) were obtained by stirring oocyst pellets with 0.5-mm glass beads until the total destruction of the oocyst walls. The pellets were suspended in 10 mM PBS buffer (pH 7.4). The beads were removed, and the lysates were sonicated twice for 30 s each time with a 5-s burst at an amplitude of 40 (Vibracell 75455; Bioblock Scientific). After centrifugation for 30 min at 15,000 × g and 4°C, the supernatant was collected, diluted to a final concentration of 1 mg/ml after bicinechonic acid (BCA) addition, and stored at −80°C. Aminopeptidase activity was tested in assay buffer (10 mM phosphate buffer, pH 7.4, 70 mM NaCl) with 40 μg of oocyst lysates using as the substrate the fluoresogenic peptide H-Ala-AMC (final concentration, 20 μM; Genecust, Luxembourg). Activities were monitored (excitation λ [λex], 350 nm; emission λ [λem], 450 nm) on a spectrophotometer (Photon Technology International). Assays were repeated three times. For inhibition assays, bestatin, 1,10-ortho-phenanthroline, or amastatin (1 to 1,000 μM) was preincubated with the lysate for 10 min before the addition of H-Ala-AMC. The residual activity was monitored as described above.

**Native zymography.** Samples were prepared in a native SDS-free and β-mercaptoethanol loading buffer before they were run on a nondenaturing, 10% acrylamide gel containing 200 μM H-Ala-AMC under native conditions (6 h, 100 V). The gel was incubated in the assay buffer at 37°C for 10 min before imaging (UV bench) using a Biot-Capt Xpress camera (Vilber Lourmat, France).

**Protein fractionation of oocysts and protease identification by mass spectrometry.** Oocysts (5 × 10⁴) were stirred with 0.5-mm glass beads until total destruction of the oocyst walls. Pellets were suspended in PBS buffer (10 mM, pH 7.4). The beads were removed, and lysates were sonicated as described above. After centrifugation for 30 min at 15,000 × g and 4°C, the supernatant was collected (final volume, 10 ml) and was subjected to isoelectric focusing using a 60-ml Rotofer cell (Bio-Rad Laboratories). The sample volume was brought to 50 ml with water, and Bio-Lyte carrier ampholytes (pH range, 3 to 10; Bio-Rad Laboratories) were added at 2.5% (wt/vol). This material was loaded into the Rotofer cell and focused at 12 W constant power for 5 h at 3°C. Twenty separate fractions were then harvested, and their respective pH values were immediately determined. The enzymatic activities of each fraction against H-Ala-AMC were monitored (final concentration, 20 μM; H-Ala-AMC, 350 nm; λem, 450 nm). Positive fractions were concentrated to a final volume of 1 ml and subjected to size exclusion chromatography using a TSK G4000PW high-pressure liquid chromatography (HPLC) column (600 by 7.5 mm; Tosoh-Haas, Cluzeau Info Labo, France) equilibrated with a 150 mM sodium phosphate buffer, pH 7.4, and an Äktapurifier 10 system (GE Healthcare, Orsay, France). Chromatography was performed at 0.5 ml/min and monitored by UV detection at 214 and 280 nm. Forty 0.5-ml fractions were collected, and active fractions were concentrated to a final volume of 1 ml. Finally, the sample was loaded on a nondenaturing 10% acrylamide gel containing 200 mM H-Ala-AMC for the native zymography assay. After migration at 4°C under nondenaturing conditions, the gel was incubated in the assay buffer at 37°C for 10 min before imaging as described above. The band of interest was excised, and protein identification was performed by mass spectrometry using an LTQ Orbitrap Velos spectrometer with an electron transfer dissociation module (Thermo Fisher Scientific) equipped with a nano-HPLC Ultimate 3000 system (Dionex) (Plate-Forme Protéomique de l’Institut Pasteur, Paris, France).

**RNA extraction and reverse transcription-PCR (RT-PCR).** Oocysts (5 × 10⁴) taken at different times after the initiation of sporulation (0 h, 12 h, 72 h) were blended with 0.5-mm glass beads until the total destruction of oocyst walls. Lysates were treated by the guanidinium thiocyanate-phenol-chloroform procedure (TRizol reagent, 1 ml; Invitrogen) for 5 min at room temperature, and total RNA was removed by 20% chloroform and precipitated with 50% isopropanol. Purified second-generation merozoites (10⁴) and gametocytes (10⁴) were directly resuspended in TRizol (1 ml; Invitrogen). RNA was suspended in nuclease-free water to a final concentration of 1 mg/ml. Aliquots of 1 μg of total RNA were used to reverse transcribe total mRNA using a poly(dT) primer and reverse trans-
cristate II (Invitrogen)). Segments of cDNA were amplified using primers specific for EtAPN1 (forward primer, 5′-GGTTTTATAGACTTCTTATAG TCTTC-3′; reverse primer, 5′-CGACCCGCAACGAGGAGCATACCCG-3′), EtAPN2 (forward primer, 5′-GGCCCGCATCGTGCCCATTTT-3′; reverse primer, 5′-GATGAGCTACTTGTGGACACC-3′), and actin (forward primer, 5′-ATCTTCATGTCAGGAGCAGCCGAC-3′; reverse primer, 5′-GTC GCCTCCACTGTTGCTCATC-3′). The conditions for PCR using GoTaq DNA polymerase (Promega) were 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 30 cycles. Amplification products were loaded onto a 1% agarose gel stained with ethidium bromide.

**Sequencing of EtAPN1.** The full sequence of EtAPN1 was amplified from an *E. tenella* unsporulated oocyst cDNA library. Primers ApN1Fwd (5′-GGTTTTATAGACTTCTTATAG TCTTC-3′) and ApN1Rev (5′-GGCTCTGGACTCACCTCA TCTTC-3′) were designed from the predicted sequence of EtAPN1 (ToxoDB accession number ETH_0001305) upstream from the predicted start site and downstream from the predicted stop codon. The EtAPN1 fragment was amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs) inserted into the pGEM-T Easy vector (Promega, France) and sequenced.

**Alignment and phylogenetic tree.** The signal peptide of EtAPN1 was predicted with the SignalP algorithm (23), and conserved protein domains were identified using the PFAM algorithm (24). The amino acid sequences of the following ETAPN family members and several selected members of the M1 zinc metalloendoproteinases were aligned using the BLAST program (GenBank accession numbers are indicated in parentheses): *E. tenella* EtAPN1 (XP_001349846.1), *Escherichia coli* APN (NP_415452.1), Saccharomyces cerevisiae Ala/Arg aminopeptidase (NP_011913.1), and Homo sapiens APN (NP_001141.2). The percent amino acid identity in the HEXH region between a given sequence and EtAPN1 was also obtained using the BLAST multialignment tool. The phylogenetic tree was computed using the MEGA (version 5) program (25) with a Muscle alignment and the neighbor-joining method. Bootstrap values were calculated after resampling 10,000 times. The predicted sequences (ToxoDB database accession numbers are given in parentheses) of *E. tenella* EtAPN2 (ETH_00015595), *T. gondii* APN3 (TgAPN3; TGME49_224460), *T. gondii* APN2 (TgAPN2; TGME49_224350), *T. gondii* APN1 (TgAPN1; TGME49_221310), Neospora caninum APN3 (NcAPN3; NCLIV_048230), and *N. caninum* APN1 (NcAPN1; NCLIV_048240) were obtained from the ToxoDB database (http://toxodb.org/toxo/).

The new sequence of *E. tenella* N1 (GenBank accession number EF602061) was used. Sequences from Cryptosporidium hominis (XP_668534.1), Cryptosporidium parvum (XP_627236.1), *P. falci- parum* (XP_001349846.1), Plasmodium vivax (XP_001617047), Plasmodium berghei (XP_680130.1), and *H. sapiens* (NP_001141.2) were obtained from the NCBI protein database (accession numbers are given in parentheses).

**Molecular modeling.** To generate a molecular model of EtAPN1, a structure-based sequence alignment was generated using the Chimeras program (26) with *E. coli* M1 as the structural template (sequence identity with the *E. coli* enzyme, 42%). The Modeler program (27) was used to model EtAPN1. The model was subjected to conjugate gradient energy minimization using the program CNS (28). Backbone and side chain atoms were subjected to harmonic restraints.

**Expression of recombinant EtAPN1.** A 3.6-kb DNA fragment encoding a partial EtAPN1 sequence (amino acids 242 to 1093) of 102 kDa was amplified from an *E. tenella* unsporulated oocyst cDNA library with primers rAPN1FwdBglII (5′-GGGAAGCTTGAAGGAGACCGACGG-GG AAGAAATTCT-3′) and rAPN1RevEcoRI (5′-GGCTCTGGACTCACCCTCA TTGCTGGCAGTGAATTGGG-3′) (the underlining indicates the BglII and EcoRI restriction enzyme sites, respectively) and Phusion High-Fidelity DNA polymerase (New England BioLabs). The expression vector pBad-EtAPN1, obtained from ligation of the insert after enzymatic restriction using BglII and EcoRI (New England BioLabs) in the pBad-HisB vector (Invitrogen), was verified by DNA sequencing. Bacteria were grown in LB at 25°C and induced with 0.002% isopropyl β-D-1-thiogalactoside (IPTG) a few hours after induction with IPTG (500 μM) at 25°C, and induced with 0.002% L-arabinose overnight.

Recombinant EtAPN1 (EtAPN1r) was purified under non-denaturing conditions following the Protino Ni-nitroliotriacetic acid agarose batch protocol (Macherey-Nagel, Germany). Purified EtAPN1r was buffer exchanged by dialysis with an 8-kDa-cutoff membrane (Spectrum Laboratory) in 1 liter of PBS overnight at 4°C. The enzymatic activity was verified using enzymatic assays with H-Ala-AMC, and the concentration was evaluated with a BCA assay (Bio-Rad Laboratories). Samples were stored at −20°C.

**Fingerprint of EtAPN1r substrates and determination of kinetic parameters.** The screening and the determination of the *K*_m, *k*_cat, and *k*_cat/*K*_m values were performed as previously described (29, 30). Briefly, EtAPN1r substrate specificity was assayed against a library of P1-substituted 7-amino-4-carbamoylmethyl coumarin (ACC) substrates, where P1 corresponds to natural and unnatural amino acids (see Table S1 in the supplemental material) (29, 30). EtAPN1r was assayed in 10 mM phosphate buffer, pH 7.4, 70 mM NaCl at 37°C. The enzyme and substrate concentrations were 30 nM and 2.5 μM, respectively. Peptidase activity was monitored (*k*_cat; 355 nm; *k*_cat/*K*_m; 460 nm), and each kinetic assay was repeated three times. The average value and standard deviation (SD) were calculated. The concentration of dimethyl sulfoxide in each experiment was less than 2%.

**Reactivation by divalent metal cations and inhibitor effects.** The ability of metal ions (Zn²⁺, Cu²⁺, Co²⁺, Fe²⁺, Mn²⁺, Mg²⁺) to restore the activity of EtAPN1r was assessed. Prior to measurement, the recombinant protein was inhibited with 1,10-ortho-phenanthroline (10 mM) for 30 min and then dialyzed with assay buffer for 30 min. After verification of the inhibition (remaining activity, 8% of the initial activity), cations (1 to 1,000 μM) were added before addition of H-Ala-AMC (20 μM). For inhibition assays, bestatin, amastatin, E64 (cysteine protease inhibitor), and 2-(aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1 to 100 μM) were incubated with EtAPN1r for 10 min before addition of H-Ala-AMC (20 μM). The activity was monitored on a spectrophotometer, and the assay was carried out three times.

**Production of polyclonal antibodies against EtAPN1r.** Rabbits were immunized with 100 μg of EtAPN1r in Freund’s complete adjuvant and boosted six times at 2-week intervals with 200 μg of EtAPN1r in Freund’s incomplete adjuvant. Total serum from rabbits was obtained by centrifugation at 850 × g for 15 min at room temperature. The specificity of polyclonal antibodies (rabbit anti-EtAPN1r [Rs-EtAPN1r] antibodies) against EtAPN1r and *E. tenella* oocyst lysates was assayed by Western blotting and compared to that for preimmune control sera.

**SDS-PAGE and immunoblotting.** Samples were prepared in an SDS-PAGE loading buffer containing 2% (vol/vol) β-mercaptoethanol and boiled for 5 min. Following migration on 10% SDS-polyacrylamide minigels, proteins were transferred to a nitrocellulose membrane (Hybond ECL; GE Healthcare). Membranes were blocked with skim milk and incubated with Rs-EtAPN1r primary antibodies (1:500). Goat antirabbit antibody coupled with horseradish peroxidase was used as the secondary antibody (1:5,000; Sigma). Immunoblots were revealed with the SuperSignal West Pico chemiluminescent substrates according to the instructions of the supplier (Pierce, Thermo Fisher Scientific) and detected using a ChemiSmart5000 apparatus (Vilber Lourmat, France).

**Immunofluorescence assay.** Freshly excysted purified sporozoites (2 × 10⁵) were incubated with 2 × 10⁵ MDBK cells in 24-well plates for various times (4 to 120 h) at 41°C in 5% CO₂. For gametes and second-generation merozoites, sporozoites were incubated with freshly prepared PCKCs as described by Hofmann and Raether (6). Cells were infected using either the *E. tenella* Wis strain (for the first step of infection and second-generation merozoite production) or the *E. tenella* Wis96 strain, which possesses only one merozoite generation and allows faster gamete formation (96 h instead of 135 h) (31). Infected cells were washed and fixed in 2.7% (vol/vol) PFA. Monolayers were then permeabilized with 0.1% (vol/vol) Triton X-100, and intracellular parasites were labeled with Rs-EtAPN1r antibody (1:500) and mouse antilasonase (Mx-Enolase) antibody (1:1000; which has constitutive expression with specificity for the
nucleus throughout the intracellular life cycle of *E. tenella* (32). Mono-
layers were incubated with a goat antirabbit antibody conjugated with
green fluorescent Alexa Fluor 488 (1:1,000; Invitrogen) and a goat anti-
mouse antibody conjugated with red fluorescent Alexa Fluor 594 (1:1,000;
Invitrogen) and mounted in Vectashield mounting medium containing
1.3 μg/ml of DAPI (Clinisciences, France) to label the nuclei. Infected cells
were examined by fluorescence microscopy (Zeiss Axiovert 200 micro-
scope; Carl Zeiss, Germany).

**Statistical analysis.** Intergroup differences (*P* values) were evalu-
ated by Student’s paired *t* test. The significance threshold was set to a *P*
value of 0.05.

**Nucleotide sequence accession number.** The EtAPN1 sequence was
submitted to GenBank with the following accession number: KF602061.

**RESULTS**

**Effects of bestatin on *E. tenella* intracellular life cycle.** To evalu-
ate the importance of metalloproteases in the *E. tenella* life cycle, we
focused on two stages: the intracellular stage with invasion and
development of sporozoites and the extracellular stage with spo-
rulation. We addressed the role of aminopeptidase activity in the
development of stable YFP⁺ sporozoites in the MDBK cell model
of infection using bestatin, a generic aminopeptidase inhibitor
(Fig. 1). After verifying the absence of bestatin cytotoxicity (see
Fig. S1 in the supplemental material), cells were incubated with
sporozoites and various concentrations of bestatin (50 to 500 μM)
for 4 h for the invasion assay and 96 h for the development assay.
In control cells, we observed the invasion of MDBK cells by spor-
zoites at 4 h and the production of merozoites at 96 h, illustrating
the expected parasitic development. The addition of bestatin did
not impair cell invasion (Fig. 1A). Conversely, bestatin signifi-
cantly reduced (*P < 0.001) the number of merozoites in the me-
dium in a dose-dependent manner. The 50% inhibitory concen-
tration (IC₅₀) of bestatin on *E. tenella* development was in the
range of 100 to 500 μM. An immunofluorescence assay was per-
formed to confirm that bestatin inhibited *E. tenella* development
but not parasite egress (Fig. 1B). Indeed, bestatin reduced the
percentage of schizonts in a dose-dependent manner, supporting
the suggestion that at least one aminopeptidase protein could play
a key role in the intracellular development of *E. tenella*.

**Detection of aminopeptidase activity during *E. tenella* spo-
rulation.** Oocyst lysates were isolated at 0, 6, 12, 24, 48, and 72 h
after the initiation of sporulation, and their aminopeptidase activity
was evaluated with a specific substrate, H-Ala-AMC. Amino-
peptidase activity was detected in each oocyst lysate during oocyst
maturity (Fig. 2A). Sporulation resulted in a significant 1.4-fold
increase of the activity at 6 h (*P < 0.001*). Then, a continuous
decrease of the hydrolysis of H-Ala-AMC was measured up to 48 h,
at which time it reached a stable value that persisted until oocyst
sporulation. In addition, preincubation of unsporulated oocyst
lysates with specific metalloprotease inhibitors (bestatin, 1,10-or-
tho-phenanthroline, and amastatin) resulted in a significant dose-
dependent reduction of aminopeptidase activity (Fig. 2B). The
IC₅₀ for bestatin inhibition of aminopeptidase activity in lysates
was comparable to that observed for inhibition of parasite develop-
ment in infected cells (Fig. 1). Similar results were obtained with
sporulated oocyst lysates (data not shown). Taken together,
the present data and the optimal pH of activity of 7.5 show that
one or several neutral aminopeptidases are active during the spo-
rulation of *E. tenella*.

**Identification of an active aminopeptidase during sporula-
tion.** Oocyst lysates isolated during sporulation were loaded on a
native acrylamide gel containing H-Ala-AMC substrate. A single
band with an apparent molecular mass of ~95 kDa was detected,
and the intensity of this band increased gradually during spo-
rulation. To identify the aminopeptidase responsible for this activity, we
performed protein fractionation of an oocyst lysate. The protease activity
was followed in parallel during the fractionation process using H-Ala-
AMC. Purification revealed that the protease possessed an isoelec-
tric point of between pH 5.2 and 5.5 and a molecular mass of
between 85 and 120 kDa (data not shown), consistent with zy-
mography results. Mass spectrometry analysis of the band, cou-
pled with searches of the sequences in the NCBI and MASCOT
Alveolata databases, identified the enzyme as a *T. gondii* amino-
peptidase N homologue (M1 family). Two putative genes were
identified in the genome sequence of *E. tenella*, and both of these
encoded aminopeptidase N proteases (EtAPNs; EtAPN1, ToxoDB
accession number ETH_00015595). Sequence analysis of these genes iden-
tified that EtAPN1 is likely the active protease. Forty-eight peptide
fragments obtained by mass spectrometry covered 56.9% of the

**Effects of bestatin on *E. tenella* extracellular life cycle.** To evalu-
ate the role of aminopeptidases in invasion (Fig. 1A) and develop-
ment (Fig. 1B) was assayed by treatment of infected MDBK cells with bestatin. For determination of invasion, cells were fixed at 4 h p.i. and the percent invasion was calculated. For determination of development, supernatants of the cell culture were collected at 96 h p.i. and the number of first-generation merozoites was estimated and compared with that for a control without inhibitor. Values are reported as the mean ± SD of three independent experiments. Intergroup differences (*P* values) between the control and treated conditions were evaluated by Student’s paired *t* test, and significance is indicated (***, *P < 0.001*). (B) MDBK cells infected with Wis YFP⁺ sporozoites were fixed at 72 h p.i. and mounted in Vectashield mounting medium. Cell nuclei were labeled with DAPI (blue). YFP⁺ parasites appear green. Images are representative of those from three individual experiments. Bar, 2 μm.
FIG 2 Metalloprotease activity is detected in *E. tenella* oocyst lysates. (A) Profile of aminopeptidase activity against H-Ala-AMC during sporulation. Protease activity from oocyst lysates was recorded at different sporulation times using H-Ala-AMC fluoropeptide. Activity was recorded after incubation of 40 µg of lysates in 10 mM phosphate buffer, pH 7.4, 70 mM NaCl at 37°C and addition of 20 µM H-Ala-AMC. Aminopeptidase activity during sporulation was normalized using the maximum activity level (observed at 6 h) as 100% peptidase activity. Intergroup differences (P values) between unsporulated (0 h) and sporulating oocysts were evaluated by Student’s paired t test, and significance is indicated (*, P < 0.001). (B) Inhibition of *E. tenella* oocyst lysate activity by aminopeptidase inhibitors. The effect of 1,10-ortho-phenanthroline (■), bestatin (□), and amastatin (■) against unsporulated oocyst lysate was assayed. The lysate was preincubated with the inhibitor before addition of the H-Ala-AMC. Values are reported as the mean ± SD of three independent experiments. Intergroup differences (P values) between the control and treated conditions were evaluated by Student’s paired t test, and significance is indicated ($, P < 0.001 for 1,10-ortho-phenanthroline; \(\ast\), P < 0.05 for bestatin; *, P < 0.001 for amastatin).

EtAPN1 full sequence. In contrast, no hit was detected for EtAPN2 (data not shown).

**Amino acid alignment of *E. tenella* aminopeptidases N with other M1 family metalloproteases members.** To investigate the similarity of EtAPN1 with other aminopeptidase N-like proteases, we cloned and sequenced the gene encoding *E. tenella* aminopeptidase N1. The coding sequence of EtAPN1 was 249 bp longer at the N-terminal end than the predicted sequence (ToxoDB accession number ETH_00013105). A signal peptide spanning amino acids 1 to 17 was predicted (P = 0.749). An alignment of amino acid sequences was generated using EtAPN2, *T. gondii* and *N. caninum* APN predicted sequences, the sequence of the M1 metalloproteases from *P. falciparum* and *C. parvum*, and the aminopeptidase N sequences of *E. coli*, *S. cerevisiae*, and *H. sapiens* (Fig. 4A). The active site of metalloproteases is characterized by two motifs: (i) the sequence HEXXHX_{6\,}E, where the His residues act to coordinate the zinc ion and the first glutamic acid residue is supposedly one of the putative nucleophiles participating in the enzymatic reaction (33), and (ii) the sequence GAMEN, which appears to distinguish aminopeptidases from leukotriene A4 hydrolase in the M1 family (34). The HEXXH motif is located between positions 510 and 533 of EtAPN1 but is not complete in the predicted sequence of EtAPN2. The GAMEN motif is also conserved in EtAPN1 and is located between positions 474 and 478. This motif is not found in the predicted sequence of EtAPN2.

The phylogenetic tree showed that EtAPN1 is closely related to the first *T. gondii* aminopeptidases N (*TgAPN1*) and aminopeptidase N1 of *N. caninum* (*NcAPN1*). It should be noted that these three coccidian parasites possess numerous aminopeptidases N, in contrast to *Plasmodium* spp. and *Cryptosporidium* spp. (Fig. 4B). Structural prediction of EtAPN1 reveals a typical bacterial alanyl aminopeptidase fold consisting of four domains (Fig. 4C) (35). The active site, zinc ion, and GAMEN motif are located in domain II. This domain is highly conserved across the M1 superfamily. The model shows good agreement with the *E. coli* enzyme (0.23 root mean square deviation [RMSD] over 4,105 atoms) and the *P. falciparum* enzyme (1.2 RMSD over 4,462 atoms). Structural variability is mainly located in the N-terminal domain and the helices of domain IV (Fig. 4C).

**Fingerprinting of EtAPN1r activity.** After production and purification of the active EtAPN1 recombinant protein (EtAPN1r), we performed inhibition assays. Figure 5 shows a significant dose-dependent reduction in EtAPN1r activity in the presence of bestatin (10 µM) and amastatin (10 µM) (P < 0.001). Conversely, no significant effect was observed with the cysteine and serine protease inhibitors E64 and AEBSF, respectively (Fig. 6A). We also investigated the requirement for a metal cofactor for enzymatic activity. EtAPN1r was first treated with 1,10-ortho-phenanthroline in order to deplete metal ions, resulting in a residual activity of about 8% compared to the initial activity. Activity was partially restored by the addition of various divalent metal cations (Zn^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+}), with Zn^{2+} being the most potent. Conversely, Co^{2+} and Cu^{2+} were poorly efficient (Fig. 6B).

We completed an EtAPN1r fingerprint to assess the P1 specificity of the aminopeptidase for N-terminal amino acids, using a library of natural and unnatural amino acids (see Table S1 in the supplemental material), as previously described (29, 30) (Fig. 7). The specificity of EtAPN1r was compared to that of PfA-M1 (29). The natural amino acid preferred by EtAPN1r is methionine, which showed 76% of MA. EtAPN1r also accommodated Ala, Arg, and Leu at P1, while Lys and Trp were cleaved at lower rates. The aminopeptidase activity against the other natural amino acids was negligible. Moreover, EtAPN1r exhibits a broad-spectrum activity against unnatural amino acids. Homophenylalanine (hPhe), norleucine (Nle), and homotyrosine (hTyr) residues were better accepted at the P1 position than 4-cyclohexyl-1-butryic acid (hCha).
norvaline (Nva), homoleucine (hLeu), homoarginine (hArg), allylglycine (allyl-Gly), and 2-aminobutyric acid (Abu). All other substrates with an unnatural amino acid at P1 had activity of less than 30% of the MA. Overall, homo-derived residues were somewhat better accepted than their corresponding encoded amino acids (hArg, 11% of the MA; hLeu, 17% of the MA; hCha, 48% of the MA; hPhe, 98% of the MA, respectively). This demonstrates that lengthening of the carbon backbone of the side chain by addition of a methylene group favors fitting within the S1 subsite. Moreover, it supports the suggestion that the S1 pocket is deep and that P1/S1 interactions are favored by bulky, aromatic residues, such as hPhe. Further, we determined kinetic parameters (the Michaelis constant \([K_m]\), catalytic constant \([k_{cat}]\), second-order rate constant \([k_{cat}/K_m]\)) of EtAPN1r against a panel of selected amino acids (Table 1). EtAPN1r showed a slightly higher affinity than PfA-M1r.
Expression and localization profile of EtAPN1 throughout the *E. tenella* life cycle. To address the biological role of EtAPN1, we first examined its expression patterns by RT-PCR on total RNA extracted from oocysts at three different sporulation times, unsporulated (0 h), partially sporulated (12 h), and sporulated (72 h), as well as second-generation merozoites and gametes (Fig. 8A). For the two aminopeptidase genes tested, EtAPN1 and EtAPN2, EtAPN1 was mainly expressed throughout the sporulation. A light EtAPN1 signal was detected in second-generation merozoites and gametes. In contrast, expression of EtAPN2 was not detected either throughout the sporulation or in second-generation merozoites or gametes.

We produced a polyclonal antibody (Rx-EtAPN1r), using the recombinant enzyme as antigen, to analyze the EtAPN1 expression profiles during sporulation (0, 6, 12, 24, 48, and 72 h) and in second-generation merozoites (Fig. 8B). The EtAPN1 gene codes a 1,093-amino-acid protein with a predicted molecular mass of 121 kDa. We immunodetected up to five forms of EtAPN1 (120, 100, 96, 68, and 38 kDa) throughout sporulation. In the unsporulated oocyst, we observed the presence of two bands corresponding to the full-length EtAPN1 (120 kDa) and an ~100-kDa form. At the end of sporulation, only the 96-, 68-, and 38-kDa forms of EtAPN1 remained. No signal was detected in second-generation merozoites. These data suggest that the 120-kDa form undergoes sequential hydrolysis into the 100-, 96-, 68-, and 38-kDa forms. These results show that EtAPN1 is synthesized and cleaved throughout sporulation.

EtAPN1 is expressed within the parasite during its intracellular development. MDBK cells and PCKCs were infected with parasites and fixed at different times. MDBK cells and Wis strain sporozoites were used for the first step of development. PCKCs were used for the production of second-generation merozoites, as first-generation merozoites are not able to invade MDBK cells. As primary culture has a limited life span, we used Wis96 strain sporozoites, which possesses only one generation of merozoites, allowing a faster formation of the gametocytes. Indirect immunofluorescence assays were performed with polyclonal Rx-EtAPN1r (Fig. 9). At 4 h, EtAPN1 was mainly detected in a punctuated manner in the sporozoite cytoplasm and was partially detected in the nucleus. At 16 h, the majority of the EtAPN1 signal colocalized with that of enolase in the nucleus, suggesting that EtAPN1 translocated from the cytoplasm to the parasite nucleus. At 24 h, the EtAPN1 signal was still detected in the parasite nucleus but was also detected in the cytoplasm. Interestingly, at 36 h of schizont development, EtAPN1 was colocalized with enolase inside the nucleus. Consistent with the findings of Western blot analysis, EtAPN1 was not detected in second-generation merozoites, while EtAPN1 was strongly immunolabeled in both macro- and microgametes, with a distinct nuclear labeling of macrogametes.

**DISCUSSION**

Aminopeptidase M1 activities have been reported in numerous apicomplexan parasites, including *Plasmodium* (36), *Toxoplasma* (37), *Cryptosporidium* (38), and *Eimeria* (13). The present results...
corroborate the previous identification of EtAPN1 as an active protease during sporulation (13). Little or no EtAPN1 expression was detected in sporozoites and merozoites, suggesting a reduced role during these intracellular stages (13). Nevertheless, based on the fact that aminopeptidases of other apicomplexa are involved in development and metabolism, the authors stated that EtAPN1 could play an important function in intracellular stages. Studies have confirmed the crucial role of the single M1-family aminopeptidase PfA-M1 in *P. falciparum* metabolism. PfA-M1 especially participates in the final steps of hemoglobin degradation and may be also involved in parasite development (10, 14, 15, 17). The present work revealed that EtAPN1, the PfA-M1 homologue of *E. tenella*, possessed a strong similarity to the plasmodial enzyme, and thus may also be involved in the development of *Eimeria*. Not only were the sequence and the three-dimensional (3D) structure similar, but also the kinetic properties toward peptidyl substrates, the maturation pattern, and subcellular localization were similar. The data suggest a strong conservation of aminopeptidase N functions in phyla besides the apicomplexa phylum as well as a critical role of EtAPN1 in the intracellular stages.

The involvement of aminopeptidase activities during *Plasmodium* species development was initially illustrated using bestatin (39), a well-known broad-spectrum inhibitor of aminopeptidases. When bestatin was added to MDBK cells infected by *E. tenella*, a strong inhibition of parasite development but not of the invasion process was observed. This suggests the presence of homologous aminopeptidases in *E. tenella* and their role in development. Previous studies (9, 10, 40, 41) indicate that at least three aminopeptidases participate in the final steps of hemoglobin degradation and may be also involved in parasite development (10, 14, 15, 17). The present work revealed that EtAPN1, the PfA-M1 homologue of *E. tenella*, possessed a strong similarity to the plasmodial enzyme, and thus may also be involved in the development of *Eimeria*. Not only were the sequence and the three-dimensional (3D) structure similar, but also the kinetic properties toward peptidyl substrates, the maturation pattern, and subcellular localization were similar. The data suggest a strong conservation of aminopeptidase N functions in phyla besides the apicomplexa phylum as well as a critical role of EtAPN1 in the intracellular stages.

**TABLE 1** Enzymatic specificity of EtAPN1a

| Substrate | $K_m$ ($\mu$M) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$·s$^{-1}$) |
|-----------|----------------|---------------------|-------------------------------|
| Nle-ACC   | 16.5 ± 2.9     | 0.710 ± 0.030       | 43.0 ± 0.2                    |
| hPhe-ACC  | 26.2 ± 8.9     | 0.836 ± 0.056       | 31.9 ± 0.2                    |
| hCha-ACC  | 23.6 ± 3.9     | 0.737 ± 0.156       | 31.3 ± 0.7                    |
| Met-ACC   | 31.4 ± 7.7     | 0.704 ± 0.180       | 22.4 ± 0.5                    |
| hArg-ACC  | 27.7 ± 2.2     | 0.575 ± 0.009       | 20.8 ± 0.1                    |
| Styryl-Ala-ACC | 30.2 ± 7.0 | 0.577 ± 0.162 | 19.1 ± 0.5                   |
| Nva-ACC   | 35.4 ± 12.5    | 0.658 ± 0.087       | 18.6 ± 0.2                    |
| Ala-ACC   | 59.8 ± 4.9     | 0.456 ± 0.033       | 7.6 ± 0.1                     |

a The kinetic parameters ($K_m$, $k_{cat}$, $k_{cat}/K_m$) of the selected substrates for EtAPN1 are shown. Values are reported as the means ± SD. Nle, norleucine.

**FIG 7** S1 substrate-binding pocket fingerprints of recombinant EtAPN1. The library consisted of 61 peptidyl substrates of the general structure H-aa-ACC (where the amino acids [aa] at P1 are natural and unnatural amino acids and ACC is the fluorescent leaving group). x axis, abbreviated amino acid (see Table S1 in the supplemental material for details); y axis, relative EtAPN1 activity expressed as a percentage of that of the best amino acid (i.e., homo-Phe). Values are reported as the mean ± SD of three independent experiments. dab, diaminobenzidine; Tip, (3L)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Bip, l-biphenylalanine; Bpa, 4-benzoyl-l-phenylalanine; Cba, l-2-amino-4-cyanobutyric acid; Igl, l-indanylglycine; met, methionyl; dhTrp, l-dihydrotryptophan.

**FIG 8** Transcription and expression profile of EtAPN1. (A) Transcription profile of *Eimeria* aminopeptidase N during the life cycle. RT-PCR was performed on total RNA collected at three different times of oocyst sporulation, unsporulated (0 h), partially sporulated (12 h), and sporulated (72 h), and from second-generation merozoites (M2) and gametes (Gam). Actin was used as an internal control. (B) Cleavage pattern of EtAPN1. Lysates obtained from oocysts isolated at different times after the initiation of sporulation (0, 6, 12, 24, 48, and 72 h) and from the merozoite lysate (M2) were assessed by immunoblotting with R-8-EtAPN1r.
tidase homologues involved in *P. falciparum* metabolism and development are present in the *E. tenella* genome (12): two aminopeptidases of the M1 family (EtAPN1 and EtAPN2) and an aminopeptidase of the M17 family (*E. tenella* leucyl aminopeptidase). Interestingly, Fetterer et al. found that EtAPN1 was transcribed in the sporulated oocyst, a relevant stage involved in earlier development steps (13). Here we showed that only EtAPN1 was transcribed during sporulation. It seems unlikely that EtAPN2 is active, as its predicted sequence lacks both GAMEN and HEXXH domains, necessary for protease activity (42, 43). Even though we cannot exclude the possibility that bestatin has side effects on other enzymes, EtAPN1 is likely the most significant target of bestatin in sporozoites. We propose that EtAPN1 is involved in parasite development and plays a role similar to that of PfA-M1. Moreover, PfA-M1 and EtAPN1 could share a still not identified function in the parasite nucleus.

It is noteworthy that *Plasmodium* and *Cryptosporidium*, in contrast to *Toxoplasma*, *Neospora*, and *Eimeria*, have only one aminopeptidase enzyme of the M1 family. Complementary phylogenetic analysis should be carried out to determine if gene duplication occurred in these parasites. Despite this difference in gene number, EtAPN1 and PfA-M1 are very closely related. Structural analysis illustrated similar 3D structures between the two enzymes, and these findings were reinforced by their similar kinetic parameters, ion requirements, and inhibition profiles.

More importantly, our data show similar biological features between the two enzymes. First, both EtAPN1 and PfA-M1 show similar patterns of cleavage of their zymogen. Western blot analysis revealed that at the beginning of sporulation, EtAPN1 is produced as 120- and 100-kDa forms. During sporulation, lower-molecular-mass forms of EtAPN1 were observed at 96, 68, and 38 kDa. This pattern is comparable to the maturation pattern of PfA-
TABLE 2 Comparison of nuclear transport components predicted in _Plasmodium falciparum_ and _Eimeria tenella_ genomes

| Protein name                  | GenBank accession no. | Annotated _E. tenella_ protein (E value) |
|------------------------------|-----------------------|-----------------------------------------|
| Importin alpha               | PF08_0087             | CDJ39306.1 (1e−127)                     |
| Importin beta                | PF08_0097             | CDJ42636.1 (1e−128)                     |
| Ran binding protein          | PF08_0095v            | AET50642.1 (3e−60)                      |
| Exportin                     | PFC0135c              | CDJ45099.1 (4e−74)                      |

*Some data are from reference 51. _Plasmodium falciparum_ nuclear transport constituents (whose accession numbers are listed in the second column) were used to perform a BLAST search of the annotated genomes of _E. tenella_. The annotated _E. tenella_ protein is listed, and the E value is given in parentheses.

M1, which is primarily synthesized as a 120-kDa precursor and is then sequentially processed as three forms of 96, 68, and 35 kDa (14, 15, 17, 36). Second, the subcellular distribution of EtAPN1 possesses features in common with that of PIA-M1. The localization of EtAPN1 is punctuated throughout the cytoplasm, in relatable agreement with the cytoplasmic localization of PIA-M1 (14–16, 36). Moreover, both EtAPN1 and PIA-M1 have an additional nuclear localization (14, 17, 44). Interestingly EtAPN1 is continuously detected in the nucleus during schizont development, suggesting a potential role inside the nucleus during schizont development and maturation. These data correlate with a previous proposal (17) for other aminopeptidases of the M1 family (45).

Interestingly, human APN/CD13 (aminopeptidase N), a zinc-binding type 2 transmembrane ectopeptidase of 150 kDa (family M1) that is selectively expressed in endothelial cells and participates in angiogenesis (46), has also been localized in the nuclear fraction (47) and is involved in cell proliferation (48, 49).

A key issue relates to the transit of EtAPN1 through the parasite nucleus. In eukaryotes, a nuclear envelope separates the nuclear and cytoplasmic compartments. Proteins with a molecular mass greater than 40 kDa cannot diffuse passively through the nuclear pore complex and require active transport through the assistance of specific transport receptors that shuttle between the nucleus and cytosol. One of these active systems involves importins/exportins that interact with the protein to be transported by a specific nuclear localization signal (NLS) (50). Using the NLS mapper tool (50) for the prediction of importin alpha interactions, we found that EtAPN1 contains an expected bipartite signal between residue 390 and residue 422, exactly 52 amino acids upstream the GAMEN motif. The calculated NLS score of 5.9 indicates that the protein can be found to be equally distributed in the nucleus and in the cytosol, which is consistent with our observation. Interestingly, a similar prediction for a bipartite importin alpha NLS sequence (residues 373 to 408) was reported for PIA-M1 at 52 amino acids upstream of the GAMEN motif (NLS score, 6.6). Moreover, it has been shown that the whole importin/exportin system is present in _P. falciparum_ (for a review, see reference 51). BLAST searches of the _E. tenella_ genome with _P. falciparum_ protein sequences identified orthologues (Table 2), supporting the suggestion that both EtAPN1 and PIA-M1 use the importin/exportin system to transit through the nucleus.

A lingering question is the difference in the signals observed between zymography and Western blot assays, as both experiments were based on the same samples. Under native conditions, variants of EtAPN1 with close molecular masses (i.e., 100- and 96-kDa forms) could migrate similarly. Therefore, a unique band corresponding to both molecules might be observed by zymography, which is the converse of the findings observed by Western blot analysis under denaturing conditions. Moreover, related peptides corresponding to both the 96- and 105-kDa forms, which are strongly expressed at 48 and 72 h, were identified by mass spectrometry (residues 150 to 1093). Conversely, the expression of the 120-kDa form of EtAPN1 was greatly decreased at 48 and 72 h, consistent with a loss of activity or signal detection by zymography.

Taken together, our results converge toward the fact that EtAPN1 and PIA-M1 possess similar functions in parasite development. Unfortunately, no knockdown or RNA interference technique to confirm the direct implication of EtAPN1 in parasite development is currently available for _E. tenella_, and the possibility that other aminopeptidases are also involved in this process cannot be excluded. Nevertheless, in the present study we clearly demonstrate the strong similarity between EtAPN1 and PIA-M1. According to the inhibition of PIA-M1 by specific bestatin-derived inhibitors that abrogate parasite development (52), we propose that nuclear EtAPN1 might also be essential for schizont development and/or maturation. Besides the usefulness of specific inhibitors in chemotherapy, the design of specific inhibitors of EtAPN1 would represent an alternative and promising strategy to investigate its role in the metabolism of _E. tenella_.

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