Proteolytic Processing of the Cryptosporidium Glycoprotein gp40/15 by Human Furin and by a Parasite-Derived Furin-Like Protease Activity

Jane W. Wanyiri,1 Roberta O’Connor,1 Geneve Allison,1 Kami Kim,2 Anne Kane,3 Jiazhou Qiu,3 Andrew G. Plaut,3 and Honorine D. Ward1*

Division of Geographic Medicine and Infectious Diseases1 and GRASP Digestive Diseases Center,2 Tufts-New England Medical Center, 750 Washington Street, Boston, Massachusetts 02111, and Albert Einstein College of Medicine, New York, New York2

Received 13 June 2006/Returned for modification 19 July 2006/Accepted 5 October 2006

The apicomplexan parasite Cryptosporidium causes diarrheal disease worldwide. Proteolytic processing of proteins plays a significant role in host cell invasion by apicomplexan parasites. In previous studies, we described gp40/15, a Cryptosporidium sp. glycoprotein that is proteolytically cleaved to yield two surface glycopeptides (gp40 and gp15), which are implicated in mediating infection of host cells. In the present study, we showed that biosynthetically labeled gp40/15 is processed in Cryptosporidium parvum-infected HCT-8 cells. We identified a putative furin cleavage site RSRR in the deduced amino acid sequence of gp40/15 from C. parvum and from all Cryptosporidium hominis subtypes except subtype 1e. Both human furin and a protease activity present in a C. parvum lysate cleaved recombinant C. parvum gp40/15 protein into 2 peptides, identified as gp40 and gp15 by size and by immunoreactivity with specific antibodies. C. hominis gp40/15 subtype 1e, in which the RSRR sequence is replaced by ISKR, has an alternative furin cleavage site (KSISKR) and was also cleaved by both furin and the C. parvum lysate. Site-directed mutagenesis of the C. parvum RSRR sequence to ASRR resulted in inhibition of cleavage by furin and the C. parvum lysate. Cleavage of recombinant gp40/15 and a synthetic furin substrate by the C. parvum lysate was inhibited by serine protease inhibitors, by the specific furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-cmk), and by calcium chelators, suggesting that the parasite expresses a Ca2+ dependent, furin-like protease activity. The furin inhibitor Dec-RVKR-cmk decreased C. parvum infection of HCT-8 cells, suggesting that a furin-like protease activity may be involved in mediating host-parasite interactions.

The apicomplexan parasite Cryptosporidium is a significant cause of diarrheal disease worldwide (16, 30). Cryptosporidiosis is asymptomatic or self-limiting in immunocompetent individuals but may be severe, chronic, and life-threatening in immunocompromised patients, such as those with AIDS. Several outbreaks of waterborne cryptosporidiosis have been reported worldwide. Because of the potential for intentional contamination of water supplies, Cryptosporidium is listed as a category B priority pathogen for bioterrorism by the Centers for Disease Control and the National Institutes of Health (27). There are 2 main Cryptosporidium species that cause human infections. Cryptosporidium hominis primarily infects humans, whereas Cryptosporidium parvum infects humans as well as other animals (33). Nitazoxanide is the only drug approved in the United States by the Food and Drug Administration for use in immunocompetent individuals with cryptosporidiosis. However, this drug is not effective against cryptosporidial infection in immunocompromised hosts (35). Therefore, the continued search for novel therapeutic interventions is critical.

Previously, we (5) and others (25, 28, 31) cloned Cpgp40/15, a highly polymorphic Cryptosporidium gene which encodes a precursor glycoprotein gp40/15 (also referred to as GP60, S60, or Cp17). This glycoprotein is proteolytically processed to yield mature glycopeptides gp40 and gp15, which remain noncovalently associated following cleavage. The C-terminal gp15 peptide is anchored in the membrane via a glycosphatidyl inositol linkage, is localized to the surface of invasive stages (sporozoites and merozoites), and is shed in trails during gliding motility (5, 9, 28, 31). gp15 (also referred to as Cp17 or S16) is an immunodominant protein consistently recognized by sera from infected persons (25). Monoclonal immunoglobulin A (IgA) antibodies to this protein are partially protective in a murine “backpack tumor” model (5), and the presence of serum antibodies to Cp17 (same as gp15) correlate with protection from diarrhea in infected humans (22). The N-terminal gp40 fragment is a secreted mucin-like glycoprotein which is present on the surface of invasive stages (most likely in association with gp15) and is also shed from these stages during gliding motility (5, 28). gp40 binds to intestinal epithelial cells, and antibodies to gp40 inhibit C. parvum infection in vitro (5). Both gp40 and gp15 contain mucin-type O-glycans that have exposed Gal(β1-3)GalNAc and/or GalNAc α1-3-Ser/Thr residues (4, 31). Lectins that recognize these residues block sporozoite attachment (4) and completely and irreversibly ablate sporozoite infectivity for host cells (10), implicating these carbohydrates in attachment and invasion. Taken together, all of these studies suggest that both gp40 and gp15 are important in mediating C. parvum infection of host cells.
The **C. parvum** gene and its products are highly polymorphic (28), particularly in **C. hominis** isolates, which cause most human infections (33). At least 8 allelic subtypes have been described among **C. parvum** and **C. hominis** isolates based on single-nucleotide and single-amino-acid polymorphisms at this locus. The finding of extensive polymorphism in the **C. parvum** locus is consistent with its gene products being surface-associated virulence determinants that may be under selective host immune pressure and indirectly supports a role for these glycoproteins in mediating infection.

Proteolytic processing is a common posttranslational modification of a number of proteins involved in attachment and invasion of apicomplexan parasites such as *Plasmodium* spp. and *Toxoplasma* spp. (reviewed in references 3, 13, and 32). Processing of these proteins occurs either during transport through the secretory pathway or after secretion onto the parasite’s surface. In many cases, proteolytic processing of these proteins has been shown to be essential for invasion of host cells by these parasites, raising the possibility that the proteases involved in processing may represent potential targets for intervention. However, proteolytic processing of *Cryptosporidium* sp. proteins has not been previously reported.

**FIG. 1.** Deduced amino acid sequence of the portion of gp40/15 surrounding the N terminus of gp15 from *C. parvum* and *C. hominis* subtypes. gp40/15 sequences from *C. parvum* (Cp) (AF155624) and *C. hominis* (Ch) subtypes 1a (AF440634), 1b (AF440626), 1c (AF440622), 1d (AF440625), 1e (AF440629), 1f (AY700389), and 1g (AY700395) were aligned using the Clustal W algorithm of the Vector NTI v8.0 program (Invitrogen). GenBank accession numbers of the sequences are underlined. The glutamic acid residue corresponding to the previously determined N terminus of gp15 is in italics.

**MATERIALS AND METHODS**

**Reagents.** The fluorogenic furin substrate, r-butyloxycarbonyl-Arg-Val-Arg-7-amino-4-methylcoumarin (Boc-RVKR-AMC) was obtained from Bachem Biosciences (King of Prussia, PA). Recombinant human furin was obtained from New England Bioslabs (Beverly, MA). The furin inhibitor decanoyl-Arg-Val-lys-Arg-chloromethylketone (Dec-RVKR-cmk) was obtained from Bachem Biosciences. The peptide-dichloromethylketone serine protease inhibitors Phe-Pro-Arg-chloromethylketone (PPACK) and Glu-Gly-Arg-chloromethylketone (GGACK) were obtained from Haematologic Technologies, Inc. (Epping Junction, VT).

**Cells.** Human ileocecal adenocarcinoma cells (HCT-8) were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete DMEM) at 37°C in 5% CO₂.

**Parasites.** *C. parvum* (Iowa isolate) oocysts were obtained from Bunch Grass Farms, Deary, ID. Prior to use, oocysts were treated with 1.75% (vol/vol) sodium hypochlorite for 10 min on ice, washed with 20 mM phosphate buffer, pH 7.2, containing 150 mM sodium chloride (phosphate-buffered saline [PBS]). Oocysts (10⁵) were excysted by incubation in 1 ml of PBS at 37°C for 1 h. The mixture of excysted oocysts and sporozoites was lysed by detergent extraction with 1% (vol/vol) Triton X-100 on ice for 1 h. The Triton X-100 lysate was adjusted to 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 7.0, 1 mM CaCl₂, 0.1% Triton X-100 (enzyme buffer) and clarified by centrifugation at 16,000 × g at 4°C for 15 min, and the supernatant (henceforth referred to as *C. parvum* lysate) was used for subsequent assays. To determine the effect of Dec-RVKR-cmk on excystation, 4 × 10⁵ hypochlorite-treated oocysts suspended in DMEM as incubated with concentrations of Dec-RVK-R-cmk ranging from 1 to 500 μM for 1 h at 37°C, and the percent excystation was calculated as follows: (number of excysted oocysts/total number of oocysts counted) × 100. The effect of Dec-RVK-R-cmk on the viability of excysted sporozoites was assessed by uptake or exclusion of the fluorogenic dyes fluorescein diacetate and propidium iodide, respectively, as described previously (2).

**Antibodies.** 4E9 is an IgM monoclonal antibody (MAb) directed against a carbohydrate epitope on gp15 (5). CrA1 is an IgA MAb directed against a protein epitope on gp15 (5). Rabbit anti-gp15 and gp15 were generated (R. M. O’Connor, J. W. Wanyiri, and H. D. Ward, unpublished data).

**Metabolic labeling of intracellular parasite proteins.** HCT-8 cells were confluent in six-well plates and infected with 4 × 10⁵ *C. parvum* oocysts per well. Twenty-four hours after infection, the culture medium was replaced with methionine/cysteine-free DMEM supplemented with 5% dialyzed FBS, with or without 1.7 μg/ml of ricin (Vector Laboratories, CA) to block host cell protein synthesis. Infected HCT-8 cells were incubated for 1.5 h at 37°C, 5% CO₂, prior to the addition of 1 μCi/ml of [3⁵S]methionine/cysteine (TRAN35SLABEL; ICN, Irvine, CA) for up to 10 min. Infected HCT-8 cells were washed once in PBS and then extracted for 30 min on ice with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.2% [wt/vol] sodium dodecyl sulfate [SDS]) containing 10 μg/ml RNase (Ambion), 20 μg/ml DNase I (Ambion), and the following protease inhibitors: 100 μM N-tosyl-l-lysine chloromethyl ketone (TLCK; Calbiochem), 100 μM (4-amidomethyl)-methane-sulfonftyl fluoride (Sigma), 2.3 mM leupeptin (Sigma), 10 μM trans-p-coumaroyl-l-leucyl-l-leucyl-l-isoleucyl-l-lysine (Calbiochem). The lysate was centrifuged at 2,000 × g for 10 min, and the supernatant was collected for immunoprecipitation.

**Immuno precipitation.** Five microliters of rabbit anti-gp40 or preimmune serum was incubated with 100 μl of the supernatant overnight at 4°C with gentle rocking, followed by incubation with prewashed protein G-Sepharose (Amer sham Biosciences, Piscataway, NJ) overnight at 4°C. After being washed (15,000 × g for 15 s) 10 times with RIPA lysis buffer, the bound immune complexes were eluted by boiling in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was treated with Autoflour (Atlanta, GA), dried, and exposed to film at −70°C.
Production and purification of recombinant gp40/15 (rgp40/15) from *C. parvum* and *C. hominis* subtype 1e. The *C. parvum* gp40/15 coding sequence was PCR amplified from *C. parvum* (GCH1 isolate) genomic DNA as described previously (5). The *C. hominis* gp40/15 subtype 1e coding sequence was PCR amplified from DNA isolated from the stool of a South Indian child infected with *C. hominis* subtype 1e (obtained as a kind gift from Sitara Rao and Gaggadeep Kang, Christian Medical College Hospital, Vellore, India) using the sense primer 5′-GGATATGGAGGGCTCAGGTTGGTCTCAGGAC-3′ and antisense primer 5′-CTGAGGATGATGTCTGTCTCGAGCTGATAGGCTCAGGACC-3′ (the mutated nucleotides are underlined) were designed using Primer X (http://biotoolsinfo.com/primers) and synthesized by Operon Biotechnologies (Huntsville, AL). Recombinant fusion proteins were purified by nickel-nitrilotriacetic acid metal affinity chromatography using Ni-NTA Superflow resin (QIAGEN, Valencia, CA) in accordance with the manufacturer’s protocol.

Site-directed mutations were designed to convert *C. parvum* gp40/15 Arg215 to Ala215. The sense primer 5′-GCGGTCGACCTCTATCGGCTCAGGAAGATCACCTCAG-3′ and antisense primer 5′-CGTGAGGATGATGTCTGTCTCGAGCTGATAGGCTCAGGACC-3′ (the mutated nucleotides are underlined) were designed using Primer X (http://biotoolsinfo.com/primers) and synthesized by Operon Biotechnologies (Huntsville, AL). Recombinant fusion proteins were purified by nickel-nitrilotriacetic acid metal affinity chromatography using Ni-NTA Superflow resin (QIAGEN, Valencia, CA) in accordance with the manufacturer’s protocol. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL).

In vitro cleavage of rgp40/15. One to two micrograms of rgp40/15 was incubated with 1 to 2 U of furin or 30 μl of *C. parvum* lysate (equivalent to 3 × 10⁶ oocysts) in a final volume of 50 μl of enzyme buffer at 37°C. For inhibition experiments, 50 mM EDTA, 10 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin, or 0.5-pH unit increments ranging from 4.0 to 8.0 using either 10% glacial acetic acid or 1 M sodium hydroxide in water as described previously (21). For determining the pH optimum for the furin-like protease activity in the lysate, the assay was performed in enzyme buffer with the pH adjusted in 0.5-pH unit increments ranging from 4.0 to 8.0 using either 10% glacial acetic acid or 1 M sodium hydroxide in water as described previously (21). For determination of the Michaelis-Menten kinetic parameters *Vₘₐₓ* and *Kₘ*, *C. parvum* lysates were treated with increasing concentrations of Boc-BVRR-AMC ranging from 1 μM to 1 mM in 100 μl of enzyme buffer in 96-well microtiter plates and incubated for 1 h at room temperature before the addition of gp40/15. The reactions were stopped by the addition of SDS-PAGE sample buffer, followed by heating at 95°C for 5 min. Cleavage products were resolved by SDS-PAGE and detected by Coomassie blue staining and Western blotting with horseradish peroxidase-conjugated S-protein (Novagen, Madison, WI) that recognizes the S tag in the recombinant protein. Identities of the cleavage products were confirmed by Western blotting with rabbit anti-rgp40 antiserum and anti-gp15 MAb GA1 (5).

N-terminal amino acid sequencing. Following resolution of rgp40/15 cleavage products by SDS-PAGE, the proteins were electrotransferred to PVDF membranes and identified by Coomassie blue staining in two separate experiments. The ~12-kDa bands from each experiment, identified as gp15 by immunoblotting with S-protein, were excised and processed for limited N-terminal sequence analysis of the first 5 amino acids by automated Edman degradation at the Tufts University Core facility.

Enzyme assays. The activities of furin and *C. parvum* lysate were monitored using the synthetic peptide fluorogenic substrate Boc-RVRR-cmk. The enzyme sample (either 1 U of furin or 1 μl of *C. parvum* lysate [equivalent to 10⁶ oocysts]) was incubated with enzyme buffer containing 1 mM Boc-BVRR-AMC in 96-well microtiter plates at 37°C for various times. For inhibition studies, the enzyme sample was preincubated with 0.1 to 5 mM concentrations of the following inhibitors at room temperature for 1 h prior to the addition of substrate: aprotinin, E64, AEBSF, pepstatin A, PMSE, TLCK, iodoacetamide, leupeptin, or 1,10-phenanthroline. The fluorescence due to released AMC was measured with a spectrophotometer (1420 VICTOR multilabel counter; Perkin-Elmer Life Sciences, Boston, MA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

To determine the pH optimum for the furin-like protease activity in the *C. parvum* lysate, the assay was performed in enzyme buffer with the pH adjusted in 0.5-pH unit increments ranging from 4.0 to 8.0 using either 10% glacial acetic acid or 1 M sodium hydroxide in water as described previously (21). For determination of the Michaelis-Menten kinetic parameters *Vₘₐₓ* and *Kₘ*, *C. parvum* lysates were treated with increasing concentrations of Boc-BVRR-AMC ranging from 1 μM to 1 mM in 100 μl of enzyme buffer in 96-well microtiter plates and incubated for 1 h at room temperature before the addition of gp40/15. The reactions were stopped by the addition of SDS-PAGE sample buffer, followed by heating at 95°C for 5 min. Cleavage products were resolved by SDS-PAGE and detected by Coomassie blue staining and Western blotting with horseradish peroxidase-conjugated S-protein (Novagen, Madison, WI) that recognizes the S tag in the recombinant protein. Identities of the cleavage products were confirmed by Western blotting with rabbit anti-rgp40 antiserum and anti-gp15 MAb GA1 (5).

Effect of Dec-RVKR-cmk on *C. parvum* infection in vitro. Hypochlorite-sterilized oocysts (5 × 10⁶/well) in DMEM were preincubated with increasing concentrations of Dec-RVKR-cmk or with DMEM alone at 37°C for 30 min and then added to confluent HCT-8 cell monolayers grown in 96-well microtiter plates. The cells were then incubated for 24 h at 37°C in 5% CO₂. Infection was quantified using an enzyme-linked immunosorbent assay-based infection assay, as described previously (5). In other experiments, HCT-8 cells were preincubated with 2, 20 or 200 μM Dec-RVKR-cmk for 30 min at 37°C and then washed 3 times with warm (37°C) complete DMEM prior to the addition of oocysts. In parallel, oocysts were treated with the same concentrations of Dec-RVKR-cmk for 30 min at 37°C and then washed 3 times with warm (37°C) complete DMEM prior to incubation with the host cells.

HCT-8 cell viability assay. HCT-8 cell monolayers were treated with various concentrations of Dec-RVKR-cmk in DMEM (without phenol red) supplemented with 10% FBS, 25 mM HEPES, 4 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and incubated for 24 h at 37°C in 5% CO₂. Viability of the cells was determined using a 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide cell proliferation assay kit (Molecular Probes, Eugene, OR).

Statistical analysis. All assays were performed in duplicate or triplicate, and the means and standard deviations were determined. All experiments were repeated at least twice. Statistical analysis was performed using GraphPad PRISM 4 software. Analysis of variance was used to compare differences in treatments.

Results

Biosynthetically labeled gp40/15 is processed in *C. parvum*-infected HCT-8 cells. Previous studies suggested that gp40/15 is proteolytically processed to yield gp40 and gp15 (5, 28). This conclusion was based on reactivity of both gp40- and gp15-specific antibodies with a ~49- to 60-kDa protein and reactivity of ~40- to 45-kDa and ~12- to 15-kDa proteins with gp40- and gp15-specific antibodies, respectively, in *C. parvum*-infected intestinal epithelial cells. To directly demonstrate processing of gp40/15, we biosynthetically labeled parasite proteins in infected HCT-8 cells with [35S]methionine/cysteine. Ricin was used to selectively block host cell protein synthesis (8). Labeled proteins were immunoprecipitated with gp40-specific antisera (gp15 contains neither methionine nor cysteine residues and, hence, cannot be labeled). Under these conditions, we were able to immunoprecipitate two proteins with relative molecular masses of ~49 and 40 kDa from infected but not uninfected HCT-8 cells (Fig. 2A). The processed 40-kDa band was detected after pulse-labeling for only 2 min (the earliest time at which incorporation of radiolabel could be detected) (data not shown), suggesting that processing occurred soon after synthesis.

Human furin cleaves recombinant *C. parvum* and *C. hominis* gp40/15 in vitro. Previous studies reported that the N terminus of the mature gp15 peptide is a glutamic acid (E) residue in the C-terminal portion of gp40/15 (Fig. 1). Examination of the amino acid sequence surrounding this residue identified an arginine-rich amino acid stretch containing a putative furin cleavage site, RRSS (Fig. 1). To determine if furin could cleave *C. parvum* gp40/15 in vitro, we cloned and overexpressed recombinant gp40/15 in *E. coli* (5) and treated the purified fusion protein gp40/15 (which migrates with the expected relative molecular mass of 52 kDa on SDS-PAGE) with recombinant human furin. This resulted in cleavage of gp40/15 into 2 fragments on SDS-PAGE with a relative molecular mass corresponding to the expected relative molecular mass of ~40 kDa for gp40 and ~12 kDa for gp15 (Fig. 2B).

This was confirmed by Western blotting with S-protein, which binds to the N-terminal S tag in the sgp40/15 and rgp40 fusion proteins but does not bind to the C-terminal sgp15 fragment (Fig. 2C). Cleavage was completely inhibited by EDTA, con-
consistent with the known requirement for Ca\(^{2+}\) by furin (21) and was also inhibited by the specific furin inhibitor Dec-RVKR-cmk (11) (Fig. 2B). Western blotting using gp40- and gp15-specific antibodies showed that the ~52-kDa and 40-kDa bands reacted with the anti-gp40 antibody (Fig. 2D) and the ~12-kDa band reacted with CrA1, an anti-gp15 monoclonal antibody (Fig. 2E). The immunoreactivity of the cleavage products with gp40- and gp15-specific antisera was consistent with that observed following cleavage of native gp40/15 (5).

Although gp40/15 is highly polymorphic in C. hominis isolates, the RSRR sequence is conserved in 6 of 7 C. hominis subtypes. However, in subtype 1e, this sequence is replaced

FIG. 2. Native gp40/15 is processed in C. parvum-infected HCT-8 cells, and recombinant gp40/15 is processed by human furin. (A) HCT-8 cells infected with C. parvum for 24 h were biosynthetically labeled with \(^{35}\)S-methionine/cysteine for 10 min. Cell lysates were immunoprecipitated with antiserum to rgp40 or preimmune serum, and the immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membranes, followed by autoradiography. Lane 1, total lysate; lane 2, immunoprecipitation with preimmune serum; lane 3, immunoprecipitation with rabbit anti-rgp40 serum. (B to F) rgp40/15 from C. parvum (B, C, D, and E) or C. hominis subtype 1e (F) was incubated with human furin at 37°C in the presence or absence of EDTA or Dec-RVKR-cmk. Cleavage products were resolved by SDS-PAGE, followed by Coomassie blue staining (B) or Western blotting with S-protein (C and F), anti-gp40 (D), and anti-gp15 (E) antibodies. Lane 1, untreated gp40/15; lane 2, gp40/15 treated with furin; lane 3, gp40/15 treated with furin and Dec-RVKR-cmk; lane 4, gp40/15 treated with furin and EDTA; lane 5, gp40/15 treated with EDTA alone.

FIG. 3. Recombinant gp40/15 is processed into gp40 and gp15 by a protease activity in C. parvum lysate. rgp40/15 was treated with C. parvum lysate for 2 h at 37°C in the presence or absence of serine protease inhibitors, EDTA, or Dec-RVKR-cmk. Cleavage products were resolved by SDS-PAGE, followed by Coomassie blue staining (A) or Western blotting with S-protein (B and C). (A) Lane 1, untreated rgp40/15; lane 2, rgp40/15 treated with C. parvum lysate; (B) lane 1, untreated rgp40/15; lane 2, rgp40/15 treated with C. parvum lysate; lane 3, rgp40/15 treated with C. parvum lysate and EDTA; lane 4, rgp40/15 treated with C. parvum lysate and AEBSF; lane 5, rgp40/15 treated with C. parvum lysate and PMSF; lane 6, rgp40/15 treated with C. parvum lysate and leupeptin; (C) lane 1, untreated rgp40/15; lane 2, rgp40/5 treated with C. parvum lysate; lane 3, rgp40/5 treated with C. parvum lysate and Dec-RVKR-cmk.
with ISKR. This sequence meets the requirement of an arginine residue at P1 and has the preferred basic residue for furin cleavage at P2 but lacks a basic residue in the P4 position. However, previous studies have shown that a basic (arginine or lysine) residue in the P2 and P6 position can compensate for an unfavorable residue at P4 (15). The subtype 1e sequence has a lysine residue at P6 and could thus potentially be cleaved by furin. To determine if this was the case, we cloned and overexpressed gp40/15 from this subtype in E. coli and treated the recombinant fusion protein with furin. As seen in Fig. 2F, this protein was indeed cleaved by furin, although the cleavage was incomplete.

A C. parvum-derived, furin-like serine protease activity cleaves recombinant gp40/15. To determine whether C. parvum expresses enzyme/s that can cleave gp40/15, we treated purified gp40/15 with a crude C. parvum lysate. Figure 3A demonstrates that the C. parvum lysate partially cleaved gp40/15 in vitro, generating fragments whose relative molecular mass on SDS-PAGE corresponded to those of rgp40 and rgp40/15. To further characterize the protease activity responsible for this cleavage, we preincubated the C. parvum lysate with the divalent cation chelator EDTA, the serine protease inhibitor AEBSF, PMSF, and leupeptin or the specific furin inhibitor Dec-RVKR-cmk before adding the gp40/15. EDTA inhibited cleavage, indicating the requirement of divalent cations for enzymatic activity. AEBSF, PMSF, and leupeptin also inhibited the cleavage of gp40/15 by the C. parvum lysate (Fig. 3B), indicating that cleavage is due to a serine-like protease activity. Furthermore, processing was inhibited by Dec-RVKR-cmk, suggesting that the protease activity has a furin-like specificity (Fig. 3C). The C. hominis gp40/15 subtype 1e was also cleaved by the C. parvum lysate (data not shown).

A mutation in the RSRR cleavage site prevents processing of gp40/15 by furin and by the C. parvum lysate. To further investigate the sequence requirement for cleavage, we used site-directed mutagenesis to generate a gp40/15 mutant by changing the P4 arginine residue to alanine (R215A). gp40/15 (R215A) was not cleaved either by furin or by C. parvum lysate (Fig. 4). These data indicate that this protease activity, like furin, requires a basic amino acid in the P4 position for activity.

Human furin and the C. parvum furin-like protease activity cleave gp40/15 at similar sites. To compare the sites at which human furin and the C. parvum furin-like protease activity cleaved gp40/15, we carried out limited amino-terminal sequence analysis on the gp15 fragment obtained after cleavage of gp40/15 with these enzymes. The N-terminal amino acid sequence of the gp15 fragment obtained following furin cleavage was 219SLSEE223, indicating that, as expected, cleavage occurred C-terminal to the P1 residue R218 (Table 1). However, the N-terminal sequence of the fragment obtained by treatment with C. parvum lysate was 223SEETS225, indicating that the parasite protease activity cleaved 2 residues downstream of the furin cleavage site (Table 1), or that the gp15 product underwent amino-terminal modification, perhaps by a C. parvum aminopeptidase.

The C. parvum protease activity is similar to that of furin in its enzyme kinetics, inhibitor profile, pH optimum, and calcium dependence. Since the C. parvum protease activity that processed gp40/15 was similar to that of furin in its substrate specificity, it was of interest to determine if this protease activity exhibited similar enzyme kinetics. We first showed that the protease activity in the C. parvum lysate could cleave Boc-RVRR-AMC (a synthetic fluorogenic substrate that is efficiently cleaved by furin) (23) in a time- and dose-dependent manner (data not shown).

We then determined the enzyme kinetics for cleavage of the Boc-RVRR-AMC peptide by the C. parvum lysate. The rate was assayed at various substrate concentrations using identical amounts of lysate. Initial velocity data were then used to establish apparent $K_m$ and $V_{max}$ values for the C. parvum protease activity. Using these data for Lineweaver-Burke analysis, we determined a $K_m$ of 15.17 ± 6.4 μM and a $V_{max}$ of 0.013 ± 0.001 μM/min. This $K_m$ value is lower than the $K_m$ of 25.9 ± 0.3 μM reported for furin (21), suggesting that the C. parvum protease activity has an even greater affinity for Boc-RVRR-AMC.

To further characterize the C. parvum protease activity, we

<table>
<thead>
<tr>
<th>Source</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum gp15 (GCH1 isolate)</td>
<td>NH₂-ETSEAA</td>
<td>5</td>
</tr>
<tr>
<td>C. parvum Cp17 (Maine isolate)</td>
<td>NH₂-ETDGAA</td>
<td>25</td>
</tr>
<tr>
<td>C. parvum Cp17 (Maine isolate)</td>
<td>NH₂-ETSEAA</td>
<td>25</td>
</tr>
<tr>
<td>C. parvum gp15 (Iowa isolate)</td>
<td>NH₂-ETSEAA</td>
<td>28</td>
</tr>
<tr>
<td>C. parvum S16 (naturally infected calves)</td>
<td>NH₂-SEETSEAA</td>
<td>31</td>
</tr>
<tr>
<td>C. parvum S16 (naturally infected calves)</td>
<td>NH₂-ETSEAA</td>
<td>31</td>
</tr>
<tr>
<td>rgp15 from human furin cleavage</td>
<td>NH₂-SLSEE</td>
<td>This study</td>
</tr>
<tr>
<td>rgp15 from C. parvum lysate cleavage</td>
<td>NH₂-SEETS</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a Deduced amino acid sequence.

*b Experimentally determined amino acid sequence.

*e. coli-expressed recombinant.
determined the effect of various classes of protease inhibitors on the hydrolysis of Boc-RVRR-AMC by the lysate. The results are shown in Table 2. Aprotinin, an inhibitor of trypsin-like serine proteases, had no effect on the furin-like protease activity, whereas other serine protease inhibitors, including AEBSF, leupeptin, TLCK, and PMSF, inhibited activity to a varying extent. In addition, the metalloprotease inhibitor 1,10-phenanthroline also significantly inhibited this activity. This profile suggests that the C. parvum lysate contains metallo-dependent serine protease activity.

To further assess the substrate specificity of the C. parvum protease activity, we investigated the inhibitory effects of various peptidylglycineleucyl dipeptide inhibitors on cleavage of Boc-RVRR-AMC by the lysate. The specific furin inhibitor Dec-RVKR-cmk, which has a pair of basic residues at P1 and P2 combined with an arginine at P4, was the most inhibitory, while both PPACK and GGACK, which contain a single arginine at P1, were poor inhibitors (Table 2). These results confirm that the C. parvum lysate contains furin-like activity.

The pH optimum for cleavage of Boc-RVRR-AMC by the C. parvum lysate was assayed in buffers of different pH values ranging from 4.0 to 8.0 and was found to be active over a broad pH range. Peak activity was observed at pH 7.0, which is identical to that of furin (21).

A direct determination of the Ca$^{2+}$ requirement of the C. parvum furin-like protease activity was not possible, since the crude lysate preparation already contains Ca$^{2+}$. We therefore determined how much EDTA was required to fully inhibit activity. As shown in Table 3, 50 mM EDTA almost completely (97%) eliminated hydrolysis of Boc-RVRR-AMC. The addition of 3 mM Ca$^{2+}$ reversed the inhibition by EDTA (Table 3). Of the divalent cations tested (Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$), only Ca$^{2+}$ was effective in reversing the inhibitory effect of the EDTA (Table 3). This is consistent with the finding that EGTA, which specifically chelates Ca$^{2+}$, also inhibits the furin-like activity of the lysate (Table 3). Since cleavage of the synthetic substrate Boc-RVRR-AMC and of gp40/15 were both blocked by the same serine protease inhibitors as well as by Dec-RVKR-cmk and EDTA, it is very likely that the protease activity in the C. parvum lysate that cleaves Boc-RVRR-AMC is responsible for processing gp40/15.

Dec-RVKR-cmk inhibits C. parvum infection of HCT-8 cells. Since gp40 and gp15 are implicated in mediating C. parvum infection, it was of interest to determine if inhibitors of proteolytic cleavage of gp40/15 had any effect on infection of host cells by the parasite in vitro. As shown in Fig. 5, the specific furin inhibitor Dec-RVKR-cmk inhibited infection of HCT-8 cells by C. parvum in a dose-dependent manner, with ~50% inhibition occurring at a concentration of 10 μM. The inhibitory effects of Dec-RVKR-cmk were not due to a toxic effect on the host cells or the parasite, since the inhibitor (at the maximal concentration used) had no effect on either host cell or parasite viability (data not shown). In addition, inhibition of

![Graph showing the effect of Dec-RVKR-cmk on C. parvum infection](https://example.com/graph.png)

**FIG. 5.** The specific furin inhibitor Dec-RVKR-cmk inhibits C. parvum infection of HCT-8 cells. HCT-8 cells were infected for 24 h with oocysts that had first been pretreated with various concentrations of Dec-RVKR-cmk (or medium alone as a control) for 30 min. Infection was quantified using an enzyme-linked immunosorbent assay-based infection assay. Each data point represents the mean ± standard deviation of results from two separate experiments, each performed in triplicate. * P < 0.01; ** P < 0.001.
infection was not due to prevention of oocyst excystation, since excystation was not altered in the presence of the inhibitor (data not shown). Inhibition of infection did not appear to be due to an effect on HCT-8 cell-derived furin, since preincubation of the cells with Dec-RVKR-cmk followed by washing of the cells prior to addition of oocysts had no significant effect on infection (data not shown). However, when oocysts were pre-treated with the same concentrations of the inhibitor and the inhibitor was washed off before the cells were infected, there was a decrease in infection, albeit not as great as when the inhibitor was present throughout the infection (data not shown), suggesting that the inhibition of infection was due to an effect on the parasite-derived furin activity rather than the host cell furin.

**DISCUSSION**

Proteolytic processing of surface and apical proteins of apicomplexans such as *Plasmodium* spp. and *Toxoplasma* spp. plays a critical role in invasion of host cells by these parasites (3, 13, 32). Here we show that the *Cryptosporidium* glycoprotein gp40/15 is proteolytically processed by human furin and by a parasite-derived furin-like protease activity, into gp40 and gp15, surface glycopeptides that are implicated in mediating parasite invasion of host cells.

Immunoprecipitation of biosynthetically labeled *C. parvum* proteins from infected HCT-8 cells confirmed that gp40/15 is processed during infection of host cells. Labeling was facilitated by using ricin to block incorporation of radiolabel into host cell proteins. This approach has been used for selective labeling of intracellular proteins of other apicomplexans, such as *Toxoplasma* and *Eimeria* (8), but has not previously been reported for *Cryptosporidium* spp. Detection of processed gp40/15 as early as 2 min after pulse-labeling suggests that cleavage is likely to occur during passage through the secretory pathway, as is the case with a number of *T. gondii* and *P. falciparum* proteins (12, 13).

The arginine-rich RSRR consensus in the gp40/15 protein sequence from *C. parvum* and all *C. hominis* subtypes except *C. hominis* subtype 1e matched a substrate consensus sequence that is recognized by subtilisin-like proconvertases such as furin (26, 29). Furin requires arginine residues at P1 and P4 and prefers lysine or arginine residues at P2, leading to our prediction that gp40/15 would be a substrate for this enzyme. Cleavage of rgp40/15 into products of the appropriate size and immunoreactivity by human furin confirmed that this protein was indeed a substrate for this enzyme. This raised the possibility that gp40/15 may be cleaved by host-derived furin, consistent with furin cleavage of other pathogen-derived virulence determinants (26). However, we also found that recombinant gp40/15 could be cleaved by protease activity present in a *C. parvum* lysate. While host-derived furin is present in the intestine (18), which is the site of infection, the finding that processing occurs soon after synthesis of the precursor supports the notion of cleavage of gp40/15 by a parasite-derived protease activity.

Site-directed mutagenesis of the furin cleavage consensus site from RSRR to ASRR resulted in complete inhibition of gp40/15 cleavage by both furin and the *C. parvum* lysate, demonstrating that, like furin, the *C. parvum* protease activity requires a basic residue at P4. Furin cleavage of the *C. hominis* gp40/15 1e in which the RSRR sequence is replaced by ISKR, indicates that the lack of a basic residue at P4 is compensated by a basic residue (lysine) at P6, as seen in other studies (14). Interestingly, although the RSRR sequence in gp40/15 is conserved in all the other subtypes, the 1e subtype is the only one that has a basic residue at the P6 position. Despite the fact that gp40/15 is highly polymorphic, the finding of conserved putative furin cleavage sites among all the known subtypes suggests that processing of this protein is functionally important.

The finding that human furin cleaved, as expected, C-terminal to the P1 arginine, whereas the N terminus of the gp15 fragment derived from cleavage by the parasite protease activity was 2 residues downstream suggests that either (i) furin and the *C. parvum* protease activity cleave gp40/15 at slightly different sites or (ii) both cleave at the same site, but the gp15 product of cleavage by the *C. parvum* protease activity is processed further by other proteases present in the lysate. The latter possibility is consistent with a previous report of a *C. parvum* aminopeptidase (24), the presence of aminopeptidase genes in the *C. parvum* genome (1), and the finding by Winter et al. (31) of four forms of native S16 (same as gp15) with different N termini (two of which are shown in Table 1). These investigators also suggested that an aminopeptidase activity of *C. parvum* might be responsible for the observed “ragged end” of the S16 (gp15) cleavage product. Additional processing following furin cleavage has also been previously reported for other substrates (18).

The *C. parvum*-derived protease activity is similar to the enzymatic activity of furin in many respects. In addition to similarities in their substrate specificity, cleavage of rgp40/15 by both enzyme activities was inhibited by the highly specific furin inhibitor Dec-RVKR-cmk. Like furin, the *C. parvum* protease activity was active across a broad pH range, with an optimum at 7.0, and is Ca\(^{2+}\) dependent. The inhibitor profile of cleavage of the furin-specific substrate Boc-RVRR-AMC by the *C. parvum* protease activity also demonstrated similarities with furin. Aprotinin, a serine protease inhibitor that has no activity against subtilisins, had no effect on the *C. parvum* protease activity, whereas the other serine protease inhibitors, PMSF, TLCK, and AEBSF, were inhibitory, albeit at relatively high concentrations. Although inhibition by PMSF and related sulfonyl fluorides are considered diagnostic for serine proteases, subtilisin-like proconvertases such as furin and Kex2 are also only inhibited by these compounds at high concentrations (21). Although the results of our study support the presence of furin-like protease activity in *C. parvum* lysates, we have no evidence at present to suggest that this activity is due to a single enzyme.

Previous studies indicated that gp40 and gp15 are important in mediating infection of host cells, raising the possibility that cleavage of gp40/15 is also essential for this process. This is supported by our finding that *C. parvum* infection of HCT-8 cells was inhibited by Dec-RVKR-cmk. Since Dec-RVKR-cmk is lipid soluble, it can penetrate cells and could thus inhibit proteolysis by parasite-derived enzymes. This inhibitor has also been shown to prevent processing of viral proteins by furin (7).

Previous studies have suggested a role for serine proteases in excystation of *C. parvum* oocysts (6, 14). In our study, the specific furin inhibitor Dec-RVKR-cmk did not affect excysta-
tion, suggesting that the infection of infection was not due to a
effect on excystation. Inhibition of infection by Dec-RVKR-
cmk also did not appear to be due to an effect on host cell furin
activity, implicating the parasite rather than the host cell pro-
etases in mediating infection. However, it remains to be
determined whether the inhibition of infection was due to an
effect on processing of gp40/15 or on that of other parasite
proteins.

Although furin or other proprotein convertases have not
been reported in apicomplexans, these parasites express sub-
tilisin-like serine proteases, including PISUB-1, PsSUB-2, and
PsSUB-3 in Plasmodium falciparum (32), TgSUB1 (19) and
TgSUB2 (20) in Toxoplasma gondii and NeSUB1 in Neospora
caninum (17). At least one gene encoding a subtilisin-like
protease has been identified in each of the Crypto-
sporidium genomes (1, 34), designated CpSUB1 for C. parvum and
ChSUB1 for C. hominis. The catalytic domains of the Crypto-
sporidium sp. subtilases have significant homology with those
of other apicomplexan subtilases as well as with bacterial sub-
tilisins and human furin (Fig. 6). However, like bacterial and
other apicomplexan subtilases, the C. parvum enzyme lack the
characteristic P domain of proprotein convertases, such as
furin and Kex2 (26). Although there is no direct evidence that
these enzymes are responsible for the furin-like protease ac-
tivity in C. parvum lysate, CpSUB1 and ChSUB1 are candidate
proteases that might cleave C. parvum or C. hominis gp40/15.

Previous reports have raised the possibility that subtilisin-
like proteases involved in processing of proteins implicated in
pathogenesis of viral, bacterial, and parasitic infections may be
potential therapeutic targets (13, 29). It is possible that, when
identified and characterized, the protease(s) responsible for
processing gp40/15 may also serve as a potential target for
intervention. However, since furin and other subtilisin-like
proprotein convertases are ubiquitous in host cells, it remains
to be determined if specific inhibitors can be designed that will

FIG. 6. The catalytic domain of CpSUB1 is homologous to that of other apicomplexan and bacterial subtilisins and to human furin. Sequences of the catalytic domains of C. parvum subtilisin CpSUB1 (AAEE01000002), Bdellovibrio bacteriovorus subtilase BsSUB (BX842649), Toxoplasma gondii subtilisins TgSUB1 (AY043483) and TgSUB2 (AF420596), Neospora caninum subtilisin NeSUB1 (AAF04257), Plasmodium falciparum subtilisins PISUB1 (AJ002233) and PISUB2 (AJ132422), and human furin (X17094) were aligned using the Clustal W algorithm of the Vector NTI v8.0 program (Invitrogen). GenBank accession numbers of the sequences are in parentheses. The catalytic triad residues for furin, aspartic acid, histidine, and serine and the putative catalytic triad residues aspartic acid, histidine, and serine for the other subtilisins are indicated by single asterisks; the oxyanion hole residue asparagine for furin and the putative oxyanion hole residue asparagine for the other subtilisins are indicated by double asterisks. Identical residues are in gray with a black background, and similar residues are in black with a gray background. The catalytic domain of CpSUB1 is 55% identical to that of B. bacteriovorus subtilase, 45% to that of TgSUB1, 45% to that of TgSUB2, 44% to that of NeSUB1, 38% to that of PISUB1, 37% to that of PISUB2, and 19% to that of human furin.
block infection by pathogens but have minimal toxicity for host cells.

In summary, we have shown that gp40/15 is proteolytically processed into gp40 and gp15 by human furin as well as by a C. parvum serine protease activity that is very similar to furin in its substrate specificity, enzyme kinetics, protease inhibitor profile, calcium dependence, and pH optimum. Further, a specific furin inhibitor abrogates C. parvum infection of host cells. Additional studies are required to definitively identify the protease or proteases responsible for processing of gp40/15 and to determine their role in mediating C. parvum infection.

ACKNOWLEDGMENTS

This work was supported by NIH grants RO1 AI05786 (to H.D.W.), RO1 DE018544 (to A.G.P.), RO1 AI46985 (to K.K.), K01 DK062816 (to R.O.), and P30 DK4392-18 and the GRASP Digestive Diseases Center at Tufts-New England Medical Center. J.W. is supported by NIH training grant T32 AI073829. G.A. is supported by NIH training grant T32 AI07389.

We thank Sitara Rao and Gagandeep Kang for C. hominis subtype 1e DNA; the Intestinal Microbiology Core of the GRASP Center for reagents, preparation of media, plasmids, and recombinant proteins; and the Tufts University Core facility for nucleotide and amino acid sequencing.

REFERENCES


Editor: W. A. Petri, Jr.