Cryptosporidium p30, a Galactose/N-Acetylgalactosamine-specific Lectin, Mediates Infection in Vitro*

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***Cryptosporidium* sp. cause human and animal diarrheal disease worldwide. The molecular mechanisms underlying *Cryptosporidium* attachment to, and invasion of, host cells are poorly understood. Previously, we described a surface-associated Gal/GalNAc-specific lectin activity in sporozoites of *Cryptosporidium parvum*. Here we describe p30, a 30-kDa Gal/GalNAc-specific lectin isolated from *C. parvum* and *Cryptosporidium hominis* sporozoites by Gal-affinity chromatography. p30 is encoded by a single copy gene containing a 906-bp open reading frame, the deduced amino acid sequence of which predicts a 302-amino acid, 31.8-kDa protein with a 22-amino acid N-terminal signal sequence. The *p30* gene is expressed at 24–72 h after infection of intestinal epithelial cells. Antisera to recombinant p30 expressed in *Escherichia coli* react with an ~30-kDa protein in *C. parvum* and *C. hominis.* p30 is localized to the apical region of sporozoites and is predominantly intracellular in both sporozoites and intracellular stages of the parasite. p30 associates with gp900 and gp40, Gal/GalNAc-containing mucin-like glycoproteins that are also implicated in mediating infection. Native and recombinant p30 bind to Caco-2A cells in a dose-dependent, saturable, and Gal-inhibitable manner. Recombinant p30 inhibits *C. parvum* attachment to and infection of Caco-2A cells, whereas antisera to the recombinant protein also inhibit infection. Taken together, these findings suggest that p30 mediates *C. parvum* infection in vitro and raise the possibility that this protein may serve as a target for intervention.*

The intestinal apicomplexan parasite *Cryptosporidium* causes human and animal diarrheal disease worldwide (1, 2) The two major species that cause human disease are *Cryptosporidium parvum,* which infects humans as well as other animals, and *Cryptosporidium hominis,* which primarily infects humans (3). Cryptosporidial infection in immunocompetent hosts is asymptomatic or self-limiting. However, in immunocompromised hosts, such as patients with AIDS, *Cryptosporidium* may cause severe, chronic, and possibly fatal disease. *Cryptosporidium* is responsible for numerous outbreaks of waterborne diarrheal disease worldwide. Because of the potential for intentional contamination of water supplies with this organism, the Centers for Disease Control have listed *Cryptosporidium* as a category B pathogen for biodefense (4). Treatment options for cryptosporidiosis are limited. Although nitazoxanide is approved by the Food and Drug Administration for use in immunocompetent individuals with the infection, this drug is not effective against cryptosporidiosis in immunocompromised patients (5).

Infection with *Cryptosporidium* occurs when oocysts are ingested with contaminated water or food or by direct person-to-person contact. Oocysts excyst in the small intestine, releasing sporozoites that attach to and invade intestinal epithelial cells. Intracellular replication occurs within a parasitophorus vacuole via asexual as well as sexual cycles. Although the pathogenic mechanisms by which *Cryptosporidium* cause disease are poorly understood, it is apparent that attachment of invasive stages (sporozoites and merozoites) of the parasite to epithelial cells and subsequent invasion of these cells are crucial events in the establishment of an infection. The ultrastructural characteristics of attachment and invasion and various factors influencing attachment have been described (6–8). However, little is known about specific parasite and host molecules involved in these processes. Knowledge of such molecules is crucial for understanding the pathogenic mechanisms involved in these interactions and for designing novel strategies to combat cryptosporidiosis. However, progress in identifying these molecules and their functional role has been severely hampered by the inability to propagate *Cryptosporidium in vitro* and to genetically manipulate the parasite (9).

In the human or animal host, *Cryptosporidium* preferentially infects small intestinal epithelial cells (10, 11). This suggests that specific parasite and/or host determinants are involved in recognition and adhesion of the parasite to host cells. A number of such intercellular recognition and adhesion functions are mediated by lectins, or carbohydrate-binding proteins, via their interaction with specific carbohydrate residues (12, 13). Lectins or carbohydrate-binding proteins have been implicated as mediators of attachment and/or invasion of host cells in a number of protozoan parasites (14).

Cryptosporidium Gal-binding Lectin, p30

Previous studies from our group reported the presence of surface-associated lectin activity in *C. parvum* sporozoites (15, 16). This activity was identified using a hemagglutination (HA)[3] assay, is optimal at a pH of 7.5 and in the presence of divalent cations Ca^{2+} and Mn^{2+}, and is most specific for the monosaccharides Gal and GalNAc (16). Gal and GalNAc-containing disaccharides and glycoconjugates, such as mucins, are potent inhibitors of lectin-induced hemagglutination. In addition, mucins block attachment to and invasion of host cells, implicating either host mucins and/or a *C. parvum* Gal/GalNAc-binding lectin in host cell recognition and adherence by *C. parvum* (17–19).

Here we describe the identification and characterization of a 30-kDa Gal/GalNAc-specific lectin named *C. parvum* or *C. hominis* protein 30 (p30), which binds to intestinal epithelial cells and mediates attachment to and subsequent infection of these cells *in vitro*.

MATERIALS AND METHODS

Parasites—*C. parvum* (GCH1) and *C. hominis* (TU502) oocysts were obtained from Dr. Saul Tzipori, Tufts University Cummings School of Veterinary Medicine (Grafton, MA), and *C. parvum* (Iowa) was obtained from Pleasant Farms, Troy, ID, or Bunch Grass Farms, Deary, ID. Oocysts were stored at 4 °C. Prior to use oocysts were treated with 1.75% (v/v) sodium hypochlorite for 10 min on ice and then washed twice with 20 mM phosphate buffer, pH 7.2, containing 150 mM sodium chloride (PBS) by centrifugation at $5000 \times g$ for 2 min at 4 °C. Hypochlorite-treated, washed oocysts were excysted for 1 h at 37 °C in the presence of 0.75% taurocholic acid in PBS. Excysted sporozoites were separated from oocysts by filtration through a 3.0-μm pore-size Nucleopore polycarbonate filter (Costar Scientific Corp., Cambridge, MA). Exocytosed or "shed proteins" were obtained as described previously (20). Soluble phase proteins were obtained by Triton X-114 extraction and phase separation. Briefly, hypochlorite-treated oocysts (1.5×10^8/ml) were excysted for 1 h at 37 °C in the presence of 0.75% taurocholic acid, and the mixture of excysted oocysts and sporozoites was incubated with ice-cold pre-condensed 2% (v/v) Triton X-114 in 10 mM Tris-HCl, pH 8.0, containing 150 mM sodium chloride (TBS-1) for 1 h on ice followed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was incubated at 37 °C for 15 min to induce phase separation. The aqueous phase containing soluble proteins (soluble phase proteins) and the detergent phase enriched in membrane proteins (detergent phase proteins) were separated by centrifugation at $1000 \times g$ for 10 min. Proteins in both phases were precipitated with 8 volumes of chilled (−20 °C) acetone overnight at 4 °C. After centrifugation at $10,000 \times g$ for 10 min, the pellet was dried at room temperature, dissolved in SDS-PAGE reducing sample buffer, heated at 95 °C for 5 min, and resolved by SDS-PAGE.

Cell Culture—Intestinal epithelial Caco-2A and HCT-8 cells were grown in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal calf sera, 25 mM HEPES, 100 units of penicillin, and 100 μg of streptomycin per ml at 37 °C in 5% CO_2 as described earlier (17).

Gal-affinity Chromatography—Hypochlorite-treated *C. parvum* oocysts (2×10^9) were resuspended in PBS containing a protease inhibitor mixture (PIC) consisting of 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin (Sigma), 20 μM pepstatin (Sigma), and 20 μM E-64 (Sigma) and excysted at 37 °C for 1 h. The resultant mixture of sporozoites and unexcysted oocysts was washed three times with PBS by centrifugation at $4500 \times g$ for 15 min, resuspended in PBS containing PIC, and lysed by sonication on ice using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT) (five times for 1 min each with an interval of 2 min, 50% duty cycle, output 3) followed by detergent extraction in 1% octyl glucoside (1% OGS) (Sigma) in PBS overnight at 4 °C, and centrifugation at 10,000 rpm for 25 min. *C. parvum* lysate or *C. hominis* soluble phase proteins were applied to Gal-agarose (EY Laboratories Inc. San Mateo, CA) columns equilibrated with PBS containing 1% OGS overnight at 4 °C. After extensive washing with PBS containing 0.1% OGS, bound proteins were eluted with 1 M Gal in 0.01% OGS/PBS. Proteins present in the eluted fractions were detected by silver staining following SDS-PAGE. Eluted fractions were pooled and concentrated using Centricon-10 centrifugal filters (Millipore Corp., Bedford, MA). Protein concentration was determined using a BCA protein assay kit (Pierce).

Gel Filtration—Gal affinity-purified proteins were separated by gel filtration on a Sephacryl S300-HR column (0.75×26.5 cm) equilibrated with TBS-1 containing 1 M Gal and 0.01% Triton X-100. 0.3-ml fractions were collected at a flow rate of 20 ml/h. The void volume (V_o) of the column was determined using blue dextran (2,000,000–4,000,000 kDa), and the column was calibrated using cytochrome *c* (14,700), carbonic anhydrase (29,000), albumin (68,000), amylase (200,000), and thyroglobulin (669,000) (all obtained from Sigma) as standards. Protein content of the fractions was monitored using a micro BCA assay kit (Pierce). Each fraction was concentrated 6-fold using Vivaspin 500 concentrators (Vivascience, Stonehouse, UK) and analyzed by Western blotting. The blots were probed with α-rp30 sera and mAb 4E9. p30, gp40, and gp900 present in the fractions were quantified by scanning densitometry of the Western blots using Quantity One software (Bio-Rad). The molecular mass of the proteins eluted in the two major included peaks was determined by the least squares line equation (21).

N-terminal and Internal Peptide Amino Acid Sequence Determination—Gal affinity-purified proteins from *C. parvum* were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore Corp.), and stained with Coomassie Blue. The 30-kDa band was excised and processed for N-terminal amino acid microsequencing by automated Edman degradation using a PerkinElmer Life Sciences ABI 477A sequencer at the Tufts University Core Facility. The N-terminal sequence of the first 11 residues was determined. To obtain

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[3] The abbreviations used are: HA, hemagglutination; Tg, *Toxoplasma gondii*; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; IFA, immunofluorescence assay; HRP, horseradish peroxidase; ORF, open reading frame; OGS, octyl glucoside; GPI, glycosylphosphatidylinositol; MALDI-TOF-MS, matrix-assisted laser desorption time-of-flight mass spectrometry.

amino acid sequence data from internal peptides, the 30-kDa band was excised from Coomassie Blue-stained SDS gels and submitted to the Harvard Microchemistry Facility, Cambridge, MA. After in-gel tryptic digestion of the protein, sequence analysis of peptides was performed by microcapillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. Four peptides were selected and analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS). These four peptides were then sequenced by Edman degradation. High confidence amino acid sequences (supported by the MALDI-TOF-MS results) were obtained for four peptides (Table 1).

Hemagglutination (HA) and HA Inhibition Assays—The presence of lectin activity was determined by using an HA assay (22). Serial doubling dilutions (in PBS containing 1 mg/ml bovine serum albumin (BSA) and 2 mM $CaCl_2$) of starting material or eluate were incubated with equal volumes of a 1% (v/v) suspension of rabbit erythrocytes in PBS in the wells of a U-bottom microtiter plate (Falcon Micro Test III™ BD Biosciences) for 1 h at 4 °C. Lectin activity was expressed (in HA units) as the reciprocal of the highest dilution of the test solution showing visible hemagglutination. Carbohydrate specificity was determined using an HA inhibition assay. Serial 2-fold dilutions of mono- and disaccharides (Sigma) in PBS were preincubated with equal volumes of test solution for 1 h at room temperature, and an equal volume of 1% rabbit erythrocytes in PBS was added. The plate was incubated for 2 h at 4 °C, and HA titer was determined as described above.

DNA Isolation, PCR, and DNA Sequencing—DNA was isolated from hypochlorite-treated, freeze-thawed GCH1 oocysts using a Fast DNA kit (Bio 101, Carlsbad, CA). Degenerate primers (P1 and P2, see Table 1) were constructed based on the amino acid sequence of the N-terminal and internal peptides shown in Table 1 (Invitrogen). Nondegenerate primers P3, P4, and P5 (Table 1) were synthesized by the Tufts University School of Medicine Core Facility. Conditions for PCR using degenerate primers were as follows: 95 °C for 2 min; 95 °C for 40 s, 37 °C for 60 s, and 72 °C for 50 s (5 cycles); 94 °C for 40 s, 55 °C for 50 s, and 72 °C for 50 s (35 cycles); and 72 °C for 5 min. The first five cycles were excluded for PCR using nondegenerate primers. Reagents were used at the following final concentrations: deoxynucleoside triphosphates, 0.4 mM; degenerate primers, 1 μM; nondegenerate primers, 0.5 μM; $MgCl_2$, 2.5 mM; and *Taq* DNA polymerase (Invitrogen). The 909-bp fragment generated by PCR using primers P3 and P5 was cloned into the pCR 2.1 vector (Invitrogen) for sequencing. Plasmids were purified using a Qiagen miniprep kit (Qiagen, Inc., Valencia, CA). DNA sequencing was performed by the dye-terminator method at the Tufts University School of Medicine Core Facility using a PerkinElmer Life Sciences ABI 377 sequencer. The BLAST algorithm was used to compare DNA and protein sequences to sequences in data bases. Analysis of nucleotide and amino acid sequences was performed using Vector NTI software (Informax, North Bethesda, MD) and the ExPASy Molecular Biology Server. The entire coding sequences of p30 were obtained from the *C. parvum* (23) and *C. hominis* (24) genome sequences using CryptoDB (25, 26). The nucleotide

sequences of p30 were deposited in GenBank™ under accession numbers AY308041 (GCH1, *C. parvum*) and AY308040 (TU502, *C. hominis*).

RT-PCR and Northern Blot Analysis—Caco-2A cells were grown to confluence in T25 tissue culture flasks and infected with *C. parvum* GCH1 oocysts (3×10^6 per flask) for 24 h at 37 °C as described previously (20). RNA was extracted from uninfected and infected Caco-2A monolayers at different time points (24, 48, and 72 h) using an RNeasy kit (Qiagen). Contaminating DNA was removed by RNase-free DNase treatment using a DNA-free™ kit (Ambion). 8 μg of total RNA was used in each RT reaction, which was performed using primers P3 and P5 (Table 1) and the Access RT-PCR System according to the manufacturer's instructions (Promega, Madison, WI). RNA from uninfected Caco-2A cells was used as a negative control. RT-PCRs without added reverse transcriptase were performed in parallel to confirm that the PCR products were not because of the amplification of contaminating DNA. The product obtained by RT-PCR was cloned into the TOPO pCR2.1 vector (Invitrogen), and the nucleotide sequence was determined as described above. As a loading control for the RT-PCRs, an 800-bp fragment of the *C. parvum* small subunit ribosomal RNA gene (SSU rRNA) was amplified using previously described primers and conditions (27).

Northern blot analysis was performed as described previously (28). Briefly, mRNA was isolated from uninfected and infected Caco-2A cells using an Oligotex QIA kit (Qiagen). 3.5 μg of mRNA was separated by 1% glyoxyl-agarose electrophoresis, transferred to a BrightStar Plus nylon membrane (Ambion) using a Northern-Gly blotting kit (Ambion) and fixed by UV radiation using a UV Stratalinker 1800 (Stratagene). The 909-bp product generated by PCR using genomic DNA (Table 1) was extracted from an agarose gel using a GEL QIAquick extraction kit (Qiagen), labeled with [^{32}P]dCTP by random priming, and the membrane hybridized with this probe. Hybridization was carried out in prehybridization/hybridization solution at 65 °C for 16 h in a PersonalHyb™ chamber (Stratagene, La Jolla, CA). The blot was washed three times with 40 mM phosphate buffer, pH 7.4, containing 0.1% SDS and exposed to film, and reactive bands were detected by autoradiography.

Expression, Purification, and Gal Binding of Recombinant p30—The p30 sequence (excluding the signal sequence) was cloned from genomic DNA and primers P4 and P5 into the pET-32 Xa/LIC vector (Novagen, Madison WI), which contains an internal factor Xa cleavage site (8 amino acids), a thrombin site (8 amino acids), an S tag (15 amino acids), a His tag (6 amino acids), and an N-terminal thioredoxin tag (109 amino acids), and the plasmid was designated pNBp30. The recombinant fusion protein designated rp30 was overexpressed in *Escherichia coli* AD494 (DE3) cells following induction with 1 mM isopropyl β-D-thiogalactopyranoside. *E. coli* cells expressing rp30 were lysed in BugBuster reagent (Novagen), and the mixture was centrifuged at 10,000 × *g* for 30 min in the presence of Benzonase 1 mg/ml (Novagen). The supernatant and pellet (containing inclusion bodies) were resuspended in TBS-1, resolved by 10% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane, and the S tag present

in the fusion protein was detected by probing with HRP-conjugated S-protein according to the manufacturer's instructions (Novagen). The majority of rp30 was detected in the pellet that contains inclusion bodies.

rp30 present in inclusion bodies was solubilized and refolded using a protein refolding kit (Novagen). The refolded protein was suspended in binding buffer (50 mM phosphate buffer, pH 7.0, containing 300 mM NaCl, 50 mM imidazole, and 5 mM CaCl$_2$), and rp30 was purified by metal-affinity chromatography using Talon metal-affinity resin (Clontech). Purity of rp30, which migrated at the expected relative molecular mass of 48 kDa, was verified by SDS-PAGE and Western blotting with HRP-conjugated S protein.

Equal amounts of purified rp30 in binding buffer were applied to two 0.5-ml Gal-agarose columns overnight at 4 °C. After washing with 10 bed volumes of binding buffer, the bound proteins were eluted with 1 M Gal or 1 M Glc in the same buffer. Eluted fractions were concentrated by ultrafiltration and analyzed by SDS-PAGE and Western blotting.

Antibodies—mAb 4E9 is an IgM mAb directed at an αGal-NAc-containing carbohydrate epitope present on *C. parvum* gp900 and gp40 (20). Antisera to purified rp30 were produced as follows. Purified rp30 was resolved by SDS-PAGE and stained with Coomassie Blue. The 48-kDa band (which was shown to represent rp30 by S tag Western blotting of a parallel strip of gel transferred to nitrocellulose) was excised and emulsified with complete Freund's adjuvant for the initial immunization and incomplete complete Freund's adjuvant for subsequent boosts. 6-Week-old BALB/c mice were immunized by intraperitoneal injection at 6–10-week intervals for a total of three immunizations, and the presence of α-rp30 antibodies in sera was monitored by Western blotting of *C. parvum* oocysts and rp30. Antisera were depleted of antibodies to rp30 by incubation with rp30 immobilized via the His tag on Talon metal affinity resin for 2 h on ice.

Western Blot Analysis—Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mAb 4E9 and α-rp30 sera as described (20). HRP-conjugated goat anti-mouse IgM and IgG (heavy and light chain) (Pierce) were used as secondary antibodies for mAb 4E9 and α-rp30, respectively. The S tag present in fusion proteins was identified in blots by reactivity with HRP-conjugated S protein according to the manufacturer's instructions (Novagen). Blots were developed with SuperSignal substrate (Pierce). Perfect Protein markers, 10–225 kDa (Novagen); were used as molecular weight standards and were detected by reactivity with HRP-conjugated S protein according to the manufacturer's protocol.

Immunofluorescence Assay (IFA)—Hypochlorite-treated oocysts (2×10^7/ml in PBS) were placed on 0.1% poly-L-lysine-coated 8-well chamber slides (Falcon), allowed to excyst at 37 °C for 1 h in a moist chamber, then air-dried, fixed, and permeabilized in methanol for 10 min at room temperature. IFAs were performed as described earlier (20, 29). Localization of p30 in intracellular stages in infected HCT-8 cells was performed by IFA as described previously (30).

Glycosidase Digestion—Hypochlorite-treated oocysts (2×10^8/ml) were excysted in the presence of 0.75% taurocholic acid for 1 h at 37 °C. The mixture of excysted oocysts and sporozo-

ites was resuspended in 50 mM Tris-HCl, pH 7.5, containing 10 mM sodium chloride (TBS-2) and PIC and lysed by freeze-thawing (20 cycles). The lysate was incubated with an equal volume of 2% Triton X-100 in TBS-2 overnight at 4 °C with gentle shaking. After centrifugation at 10,000 × g for 30 min, the supernatant was treated with recombinant peptide *N*-glycosidase F, 300 units/ml (New England Biolabs, Beverley, MA), or α-*N*-acetyl-galactosaminidase, 500 units/ml (New England Biolabs), at 37 °C overnight according to the manufacturer's instructions. As a control for these glycosidases, aliquots of lysate were treated with buffer alone under identical conditions.

p30 Binding Assay—Binding of p30 to Caco-2A cells was assessed by ELISA (20) and IFA. Soluble phase proteins were used as a source of p30. To quantify binding by ELISA, Caco-2A cells were grown to confluence in 96-well plates and fixed with 1% glutaraldehyde in PBS for 10 min at room temperature. After three washes with PBS, nonspecific binding was blocked with PBS containing 1% BSA (blocking buffer-1) for 2 h at room temperature. Serial doubling dilutions of the soluble phase proteins (starting concentration 500 μg/ml) were preincubated with equal volumes of PBS or 1 M Gal in PBS for 1 h at room temperature and then added to the glutaraldehyde-fixed Caco-2A cells. The plate was incubated for 2 h at 37 °C. Unbound proteins were washed off with PBS and bound proteins fixed with methanol for 10 min at room temperature. After washing three times with PBS, nonspecific binding was blocked with TBS-1 containing 0.1% BSA (blocking buffer-2) for 1 h at room temperature and then incubated with α-rp30 sera diluted 1:500 in blocking buffer-2 overnight at room temperature. The plate was washed three times with TBS-1 and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted to 2 μg/ml in blocking buffer-2. After washing three times with TBS-1, *p*-nitrophenyl phosphate substrate (Sigma) in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl$_2$ and 5 mM MgCl$_2$ was added, and absorbance at 405 nm was read using a Bio-Rad model 550 plate reader (Bio-Rad). β-Galactosidase (detected using anti-β-galactosidase mAb Gal-13 (Sigma)) was used as a negative control. rp30 or a control recombinant protein containing only the fusion tags (31), both at a final concentration of 100 μg/ml, were preincubated for 1 h at room temperature with or without serial doubling dilutions of Gal and GalNAc or disaccharides containing them, and binding was assessed in a similar manner by ELISA using α-rp30 sera that also recognizes the fusion tags in the control recombinant protein (not shown).

To detect p30 binding by IFA, Caco-2A cells were grown to confluence in 8-well chamber slides (Falcon, Franklin Lakes, NJ) and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. After washing three times with PBS, nonspecific binding was blocked with blocking buffer-1 for 1 h at room temperature. Soluble phase proteins (200 μg/ml) preincubated for 30 min at room temperature with equal volumes of 2 M Gal in PBS or PBS alone were incubated with the cells overnight at 4 °C. Unbound proteins were washed off with PBS and bound proteins fixed with methanol for 10 min at room temperature. After washing three times with PBS, nonspecific binding was blocked with blocking buffer-1 for 1 h at room temperature and then incubated with α-rp30 sera diluted 1:200 in blocking buff-

er-1 for 1 h at room temperature. The slide was washed three times with PBS and incubated with Alexa Fluor 488 fluorescein isothiocyanate-labeled anti-mouse IgG (Molecular Probes) diluted 1:100 in blocking buffer-1. After washing three times with PBS, slides were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories).

Attachment Assay—Caco-2A cells were grown to confluence in 96-well plates and fixed with 1% glutaraldehyde as described above. After washing three times with PBS, cells were incubated with rp30 or the control recombinant protein at increasing concentrations for 30 min at room temperature. Sporozoites (5×10^4/well) were then added and plates incubated at 37 °C in 5% CO_2 for 1 h. Unbound sporozoites were removed by washing with pre-warmed DMEM without serum and cells with attached sporozoites were fixed in methanol for 10 min at room temperature. Sporozoite attachment was quantified by ELISA using mAb 4E9 as primary antibody at a dilution of 1:3000 in blocking buffer-2 as described previously (20).

Infection Assay—To investigate the effect of α-rp30 sera on *C. parvum* infection, oocysts (2×10^4/well) were preincubated with equal volumes of α-rp30 sera or nonimmune mouse sera at different dilutions (1:25–1:200) in DMEM without serum at 37 °C for 30 min and then added to Caco-2A cells grown to confluence in 96-well plates. To determine the effect of rp30 on infection, Caco-2A cells were preincubated with increasing concentrations of rp30 for 30 min at room temperature. Oocysts (2×10^4/well) were then added to the wells and plates incubated at 37 °C in 5% CO_2 for 24 h. After washing with pre-warmed DMEM, cells were fixed with methanol for 10 min at room temperature, and the infection was quantified by ELISA as described above.

Statistics—All assays were performed in 2–4 replicates and experiments repeated 2–5 times. Results were expressed as the mean ± S.E. Statistical comparisons were performed with the 2-way analysis of variance test using the Graph Pad Prism program (Graph Pad Inc., San Diego). p values <0.05 were considered significant.

RESULTS

Identification of Gal-binding Proteins by Gal-affinity Chromatography—The carbohydrate specificity of the sporozoite lectin activity was exploited to isolate putative Gal/GalNAc-binding proteins from *C. parvum* lysate or *C. hominis* soluble phase proteins by Gal-affinity chromatography. Analysis of the affinity-purified proteins from both species by SDS-PAGE and silver staining revealed the presence of two bands, a major band of 30 kDa and a second very high molecular weight band of >225 kDa (Fig. 1, *a* and *b*). The high molecular weight band was identified as gp900 by reactivity with 4E9 (Fig. 1*c*), a monoclonal antibody (mAb) against a GalNAc-containing carbohydrate epitope (20). mAb 4E9 also reacted with 49- and 40-kDa bands (although these were not visualized by silver staining). These bands represent the mucin-like glycoprotein gp40/15 and its proteolytic cleavage product gp40 (31), which are not readily identified by silver staining in small quantities.[4]

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[4] N. Bhat, A. Joe, M. PereiraPerrin, and H. D. Ward, unpublished observations.
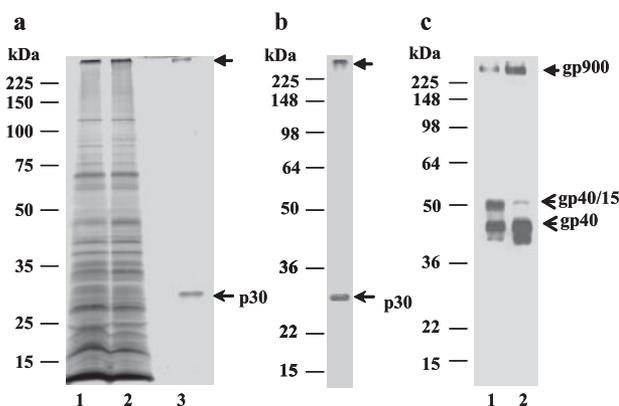
FIGURE 1. **Identification of Gal-binding proteins by Gal-affinity chromatography.** *a* and *b*, *C. parvum* sporozoite lysate (*a*) or *C. hominis* soluble phase proteins (*b*) were applied to a Gal-agarose column, washed, and eluted with 1 M Gal. Aliquots of starting lysate (*a, lane 1*), effluent (*a, lane 2*), and Gal eluate (*a, lane 3*, and *b*) were separated by 5–15% gradient SDS-PAGE and bands visualized by silver staining. *c,* eluted proteins from *C. parvum* (*lane 1*) or *C. hominis* (*lane 2*) were separated by 5–15% gradient SDS-PAGE, electrotransferred to nitrocellulose, and probed with mAb 4E9.

4E9 did not react with the 30-kDa band. To investigate the identity of this protein, we determined its N-terminal amino acid sequence (Table 1). Comparison of this sequence with the deduced amino acid sequences of gp900 and gp40/15 revealed no similarity, indicating that the proteins are not related.

Carbohydrate Specificity of Gal-binding Proteins—To determine whether the Gal affinity-purified material displayed lectin activity, we employed an HA assay following extensive dialysis to remove Gal. The affinity-purified material from both species had an ∼200-fold increase in lectin-specific activity when compared with the crude starting material (Table 2). We determined the carbohydrate specificity of the Gal affinity-purified material by HA inhibition using a panel of monosaccharides. GalNAc and Gal were the most potent inhibitors of lectin activity (Table 3), which is consistent with the previously identified carbohydrate specificity of the lectin activity from crude sporozoite lysates (16).

Cloning and Expression of the Gene Encoding p30—To clone the gene encoding p30, degenerate PCR primers were designed on the basis of the amino acid sequences of the N-terminal and internal tryptic peptides of the *C. parvum* 30-kDa band excised from SDS gels (Table 1). Using degenerate primers P1 and P2 (Table 1), a 315-bp fragment was PCR-amplified from *C. parvum* genomic DNA (Fig. 2*a*). BLAST comparison of the nucleotide sequence of this fragment with the *C. parvum* (23) and *C. hominis* (24) genome sequences using CryptoDB (25) identified 2251-bp contigs with 99.8% similarity to each other. These sequences contained 909-bp, single copy, open reading frames (ORFs) designated *C. parvum* and *C. hominis p30*, respectively. Both ORFs were then PCR-amplified from *C. parvum* (GCH1 isolate) and *C. hominis* (TU502 isolate) genomic DNA and cloned into the TOPO pCR2.1 vector, and the nucleotide sequence of two independent clones from each was obtained. Sequence analysis revealed 98.9% similarity between the *C. parvum* and the *C. hominis* sequences (data not shown). Analysis of the deduced amino acid sequence of the p30 ORF (Fig. 2*a*) revealed a 302-amino acid protein with a predicted relative molecular mass of 31.9 kDa and a pI of 6.5.

TABLE 1

Sequence of p30-derived peptides and synthetic oligonucleotides used for PCR and RT-PCR

Peptides	Oligonucleotides
NGVC1LKKG[a]	P1, 5′–AATGGAGUCTGCATTCUUAAAAAAGG–3′ (Forward)
FIAGSKV	
AYSLYK	P2, 5′–TTTATAUUGGGUATAAGC–3′ (Reverse)
QEWLATSSGSFVGR	–
FIYQSVSQGTMNVSR	–
	P3, 5′–ATGAGCGGCGCGCGCAGCAGCATTTTTATT–3′ (Forward)
	P4, 5′–AACGGCGTGTGCATTCTGAAAAAAGGCGC–3′ (Forward)
	P5, 5′–ATAGCTGTTCATGGTGCCCTGGCTC–3′ (Reverse)

[a] This is an N-terminal peptide; P1 and P2 are degenerate primers.

TABLE 2

Specific lectin activity of Gal-affinity purified material from *C. parvum* and *C. hominis*

	Volume	Protein	Lectin activity (HA titer)	Specific activity (HA titer/ mg protein)	-Fold increase in specific activity
	ml	*mg*			
C. parvum starting material	16	31.49	7935	252	0
C. parvum eluate	0.042	0.0198	1078	54468	216
C. hominis starting material	1	15.5	4049	261	0
C. hominis eluate	0.05	0.0351	1762	50,196	192

TABLE 3

Monosaccharide specificity of Gal-affinity purified proteins from *C. parvum* and *C. hominis*

Monosaccharides	*C. parvum*		*C. hominis*	
	MIC[a]	RIC[b]	MIC	RIC
Gal	6.2	1	6.2	1
GalNAc	3.1	2	3.1	2
Glc	100	0.062	>100	0.062
GlcNAc	50	0.124	>100	0.062
Man	>100	<0.062	>100	<0.062
Fuc	>100	<0.062	>100	<0.062

[a] Minimal inhibitory concentration (MIC) (in mM) was determined by HA inhibition.
[b] Relative inhibitory capacity (RIC) was compared with Gal.

a

P1
315 bp
P2

911   1852   2251 bp
P3
906 bp ORF
P5

b
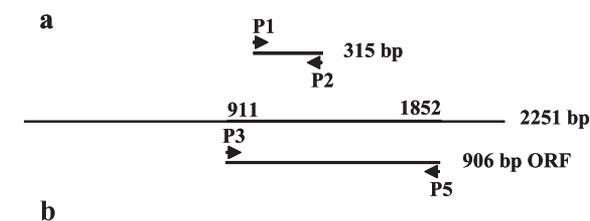
*MSGARSSIFIACILYFISLVSA*NGVCILKKGASCLN**AS**YSFIEGGMTPGDAI
RVSVVLLPSASLELTN**SS**FQNIATLRFGVDSCSIESGIASLRAYSLYKNGQ
YNTGSTISFDLVALEKSLAIFIAGSKVLEIPLLVAAPFMLTSDSN**GSA**PTL
QFLEYSTTETGFCSIASSGSCN**NSS**LVGMSNGVTKN**LTI**RAELPSSIPDDY
FSFYISTYSNIYKLPVLEVAFTK**QEWLATSSGSFVGR**GKIPAGINLGSSINF
VLVPSSGGSVELKVNEKSLGVVKVDASSIKFIYQSVSQGTM**N**VSY

FIGURE 2. **Cloning and analysis of the *p30* gene.** *a*, sequential PCR steps used to clone the *p30* gene. Primers are defined in Table 1. *b*, deduced amino acid sequence of *p30*. The signal peptide sequence is in *boldface* and *italics*. N-terminal and internal peptides identified by MS/MS analysis are *underlined*. Six predicted *N*-glycosylation sites are in *boldface*.

The amino acid sequence corresponding to the N terminus of the native p30 was found 22 amino acids after the start codon. This N-terminal hydrophobic 22-amino acid stretch (Fig. 2*b*) was predicted to be a signal peptide using the SignalP 3.0 server. The amino acid sequences of all four peptides determined by amino acid sequencing of internal tryptic peptides of

a

kB

0.9 — p30

0.8 — SSU rRNA

1 2 3 | 4 | 5 6 7 8 9 10 | 11 | 12
Uninfected   Infected   gDNA

b

kB

2.0 —
1.5 —
1.0 —
0.5 —

1   2
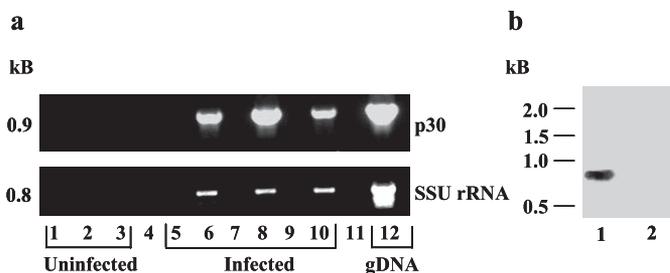
FIGURE 3. **p30 is expressed within 24–72 h after *C. parvum* infection.** *a*, RNA was extracted from uninfected Caco-2A cells after 24 h (*lane 1*), 48 h (*lane 2*), and 72 h (*lane 3*) and from infected Caco-2A cells after 24 h (*lanes 5* and *6*), 48 h (*lanes 7* and *8*), and 72 h (*lanes 9* and *10*) with *C. parvum* and analyzed by RT-PCR using gene-specific primers P3 and P5 (see Table 1). RT-PCRs in *lanes 5, 7,* and *9* were performed without reverse transcriptase. Genomic (*g*) DNA was used as positive control (*lane 12*). *Lanes 4* and *11* are blank. *b*, Northern blot analysis of *p30* transcript. mRNA from *C. parvum*-infected (*lane 1*) or -uninfected (*lane 2*) Caco-2A cells was extracted after 24 h of infection, subjected to Northern blotting, and probed with a *p30* probe.

the native *C. parvum* protein were also identified in sequences from both species (*underlined* in Fig. 2*b*). There were no predicted transmembrane domains or putative glycosylphosphatidylinositol (GPI) anchor attachment sequences. Six *N*-glycosylation sites were predicted using the NetNGlyc 1.0 server, whereas no predicted *O*-glycosylation sites could be identified using the NetOGlyc 3.1 server. BLAST searches did not reveal significant similarity of *p30* with any known genes in data bases, including the genomes of the related apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii* (32).

p30 Is Expressed 24–72 h after C. parvum Infection of Intestinal Epithelial Cells—Time course studies showed that expression of the *p30* gene was detected at 24, 48, and 72 h after *C. parvum* infection of Caco-2A cells but not in uninfected cells (Fig. 3*a*). Although the analysis was semi-quantitative, compared with the expression of the *C. parvum* small subunit ribosomal rRNA gene, *p30* expression appeared to be maximal at 48 h suggesting that it may be regulated. Northern blot analysis of mRNA from Caco-2A cells infected for 24 h with *C. parvum* revealed a single ~1-kb transcript in mRNA from infected but not uninfected Caco-2A cells (Fig. 3*b*).

Expression, Purification, and Gal Binding Activity of Recombinant p30—Recombinant p30 (rp30) was overexpressed in *Escherichia coli*, and the fusion protein was purified by metal affinity chromatography (Fig. 4*a*). To determine whether rp30 had Gal binding activity, equal amounts of the purified protein were applied to two Gal-agarose columns, which were washed and eluted with an excess of either Gal or Glc. The finding that rp30 was eluted with Gal (Fig. 4*b*) but not with Glc (Fig. 4*c*), suggests that rp30 binds specifically to Gal. However, rp30 did
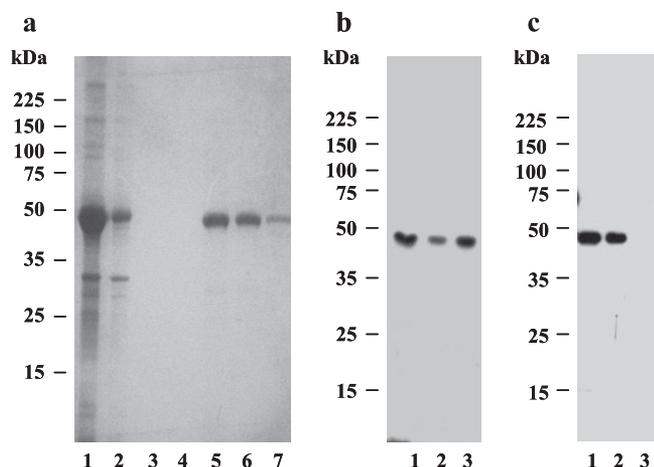
FIGURE 4. **Purification and Gal binding of recombinant p30.** *a,* rp30 was purified by metal affinity chromatography, and aliquots of the starting lysate (*lane 1*), flow-through (*lane 2*), washes (*lanes 3* and *4*), and eluate (*lanes 5–7*) were analyzed by 10% SDS-PAGE and staining with 0. 1% Coomassie Brilliant Blue. *b* and *c,* purified rp30 was applied to two Gal-agarose columns, and washed and bound proteins were eluted with either 1 M Gal (*b*) or 1 M Glc (*c*). Aliquots of purified rp30 (*lane1*), flow-through (*lane 2*), and eluate (*lane 3*) were analyzed by Western blotting with S protein.
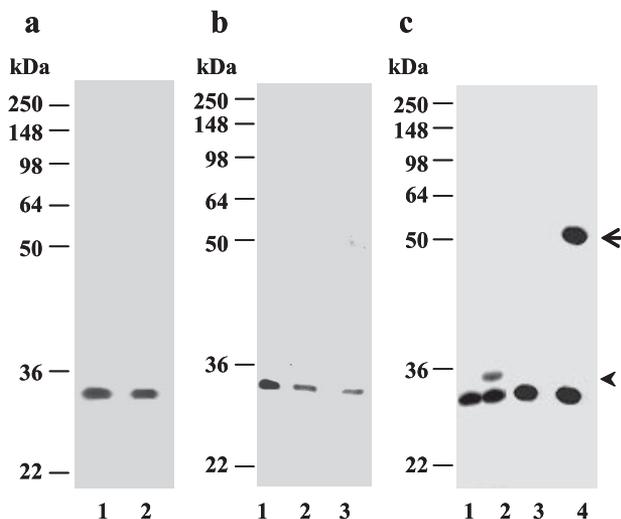
FIGURE 5. **p30 is expressed in oocysts, sporozoites, and exocytosed proteins and does not appear to be glycosylated.** *a* and *b,* aliquots of *C. parvum* (*a, lane 1,* and *b, lane 1*) or *C. hominis* oocysts (*a, lane 2*), sporozoites (*a, lane 2*), and exocytosed proteins (*b, lane 3*) were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with α-rp30 sera. *c, C. parvum* lysate was treated with buffer alone (*lanes 1* and *3*) or with peptide:*N*-glycosidase F (*lane 2*) or α-*N*-acetylgalactosaminidase (*lane 4*). Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with α-rp30 sera. The bands indicated with the *arrowhead* and *arrow* most likely represent nonspecific reactivity with the enzymes peptide:*N*-glycosidase F (36 kDa) and α-*N*-acetylgalactosaminidase (47 kDa), respectively.

not display HA activity with rabbit erythrocytes (not shown) suggesting that it does not form dimers or oligomers.

p30 Is Present in Oocysts, Sporozoites, and Exocytosed Proteins and Does Not Appear to be Glycosylated—Antisera raised in mice to purified rp30 (designated α-rp30) reacted with the rp30 fusion protein (not shown) and with an ∼30-kDa band in *C. parvum* and *C. hominis* oocysts (Fig. 5, *a* and *b*) by Western blotting. α-rp30 also recognized an ∼30-kDa band in *C. parvum* sporozoites as well as exocytosed/secreted proteins (Fig. 5*b*) indicating that p30 is expressed in oocysts and sporozoites and is exocytosed or secreted from them.
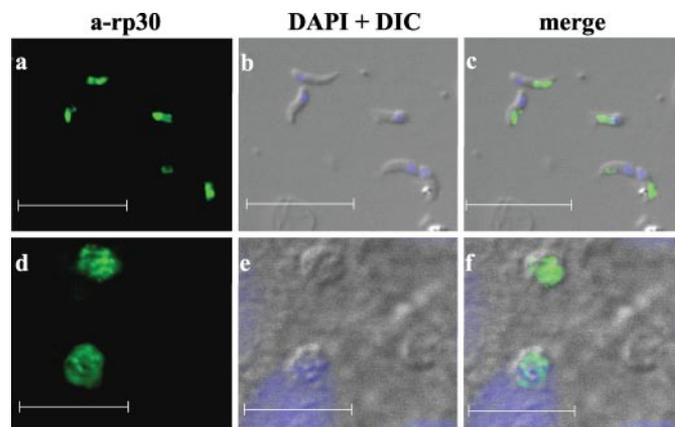
FIGURE 6. **p30 localizes to the apical region of sporozoites and is predominantly intracellular in sporozoites and intracellular stages.** Excysted oocysts and sporozoites (*a– c*) or HCT-8 cells infected with *C. parvum* for 24 h (*d–f*) were fixed with methanol and analyzed by IFA using α-rp30 sera. There was no reactivity with preimmune sera or sera depleted of α-p30 antibodies (not shown). *Bar* = 10 μm; *DAPI,* 4′,6-diamidino-2-phenylindole; *DIC,* differential interference contrast.

Analysis of the deduced amino acid sequence of *p30* revealed six predicted *N*-glycosylation sites but no predicted *O*-glycosylation sites. To determine whether p30 was *N*- or *O*-glycosylated, we treated *C. parvum* proteins with peptide *N*-glycosidase F (which cleaves *N*-linked glycans) and with α-*N*-acetylgalactosaminidase (which cleaves terminal α1–3GalNAc or GalNAcα 1-Ser/Thr from *O*-linked glycoproteins) and analyzed the treated proteins by SDS-PAGE and Western blotting with α-rp30 sera. There was no change in relative molecular mass of p30 with either treatment (Fig. 5*c*), suggesting that either p30 is not *N*- or *O*-glycosylated or that glycosylation if present is not sufficient to result in a decrease in relative molecular mass following enzymatic deglycosylation.

p30 Localizes to the Apical Region of Sporozoites and Is Predominantly Intracellular in Both Sporozoites and Intracellular Stages—We used the α-rp30 sera to determine the localization of p30 in methanol-fixed sporozoites and intracellular stages. This indicated that the protein was localized to the apical region of sporozoites and was predominantly intracellular in both sporozoites and merozoites within meronts (Fig. 6). However, localization to specific intracellular organelles could not be determined by light microscopy and will need to be ascertained by immunoelectron microscopy. Preimmune sera and antisera depleted of α-rp30 antibodies did not react with sporozoites indicating specificity of the reactivity (data not shown).

p30 Associates with gp900 and gp40 and Is a Gal-binding Lectin—The finding that p30 co-purified with gp900 and gp40 by Gal-affinity chromatography suggested that either each of these proteins are Gal-binding lectins or that they are noncovalently associated with each other, possibly via binding of p30 to Gal/GalNAc residues that are known to be present on gp900 and gp40 (20). To investigate these possibilities, we first performed Triton X-114 extraction and phase separation on a mixture of excysted oocysts and sporozoites to determine whether these proteins partitioned into the same phase. gp900 is an integral membrane protein (33), and gp40/15 is anchored in the membrane via a GPI anchor (34). Once it is cleaved from gp40/15, gp40 remains associ-

ated with the GPI-anchored gp15 fragment and partitions into the detergent phase following Triton X-114 extraction and phase separation (35). We reasoned that if p30, which is a secreted soluble protein, associates with gp900 and/or gp40/15 and gp40, they should all partition into the detergent phase. As expected, most of gp40/15 and gp40 partitioned into the detergent phase (Fig. 7*b*). Surprisingly, however, p30 as well as most of the gp900 partitioned into the soluble phase (Fig. 7*a*). This may be due to proteolytic cleavage (most likely) or "shedding" of gp900 from the parasite surface (33). However, the finding that both p30 and gp900 partitioned into the same phase suggests that they do associate with each other possibly via binding of p30 to Gal/GalNAc residues on gp900.

To investigate this possibility, we next attempted to separate the proteins by gel filtration chromatography in the presence of an excess of Gal. We used the α-rp30 sera and mAb 4E9 to quantify p30 and g900 and gp40, respectively, by scanning densitometry of immunoblots of each of the eluted fractions (Fig. 8, *a* and *b*). gp900 and gp40 eluted in the void volume peak (V_o), whereas p30 was eluted in two major included volume peaks (V_i1 and V_i2), which corresponded to molecular masses of 63 and 126 kDa, respectively (Fig. 8*c*). To determine which protein(s) exhibited lectin activity, we dialyzed the pooled peaks extensively to remove Gal and performed an HA assay using rabbit erythrocytes. The void volume peak did not exhibit HA activity, whereas the included volume peaks had HA titers of 128 and 64 (with lectin specific activities of 94,117 and 66,661), respectively. Taken together these results suggest the following: 1) native p30 exists as dimers or tetramers consisting of identical subunits; 2) p30 may associate with gp900 and gp40 by binding to Gal/GalNAc residues on these proteins; 3) native gp40 may exist in multimers or may associate with gp900 via non-Gal-binding interactions; and 4) p30 and not gp900 or gp40 is responsible for the lectin activity of the Gal affinity-purified material.

Native and Recombinant p30 Bind to Intestinal Epithelial Cells via Gal/GalNAc-binding—Because p30 is a Gal/GalNAc-specific lectin, it was of interest to determine whether this protein binds to host cells. To determine this, we incubated soluble phase *C. parvum* proteins that are enriched in p30 (Fig. 7*a*) with intestinal epithelial Caco-2A cells and detected p30 binding by ELISA and IFA using α-rp30 sera. The results indicated that binding of p30 (but not the control protein, β-galactosidase) occurred in a dose-dependent and saturable manner as measured by ELISA (Fig. 9*a*). These findings were confirmed by IFA (Fig. 9*b*). In both assays, binding could be inhibited by an excess of Gal, suggesting that
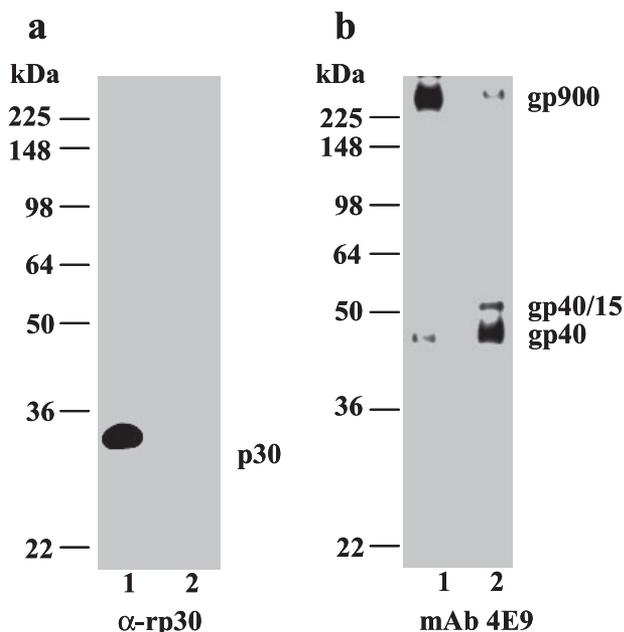
FIGURE 7. **Triton X-114 phase separation of p30, gp900, and gp40.** *a* and *b*, *C. parvum* lysate was subjected to Triton X-114 extraction and phase separation. Aliquots of aqueous phase proteins (*lane 1*) and detergent phase proteins (*lane 2*) were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with α-rp30 sera (*a*) and mAb 4E9 (*b*).
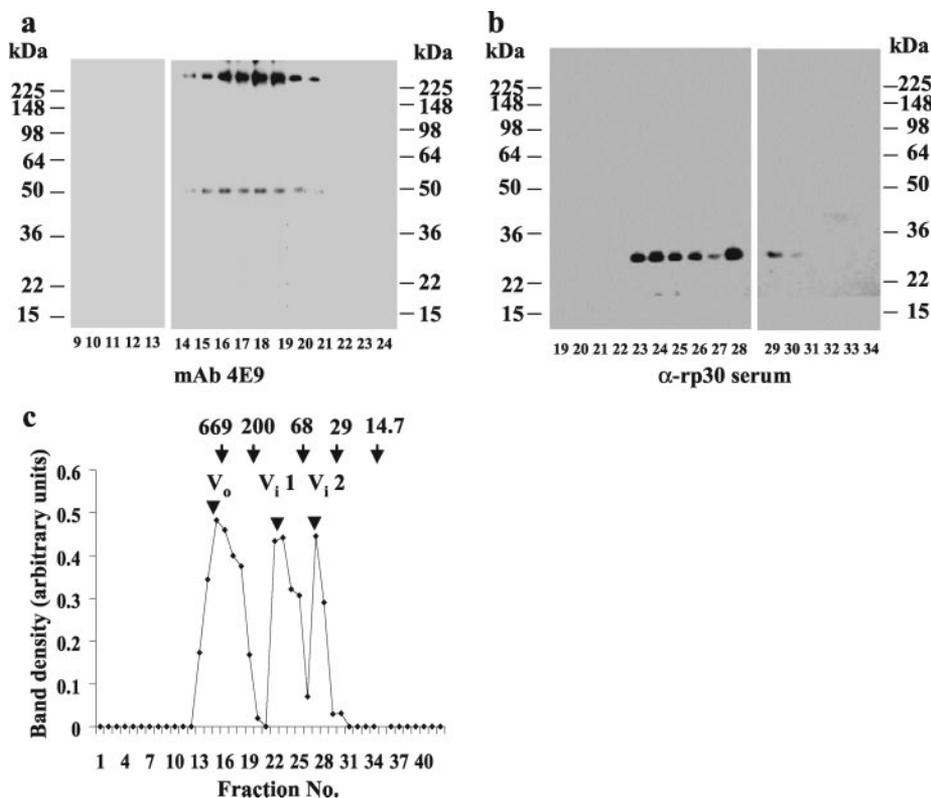
FIGURE 8. **Separation of p30, gp900, and gp40 by gel filtration.** *a–c,* Gal affinity-purified material was applied to a Sephacryl S-300-HR column in the presence of 1 M Gal, and 0.3-ml fractions were collected (*c*). p30, gp900, and gp40 present in the fractions were quantified by scanning densitometry of Western blots probed with mAb 4E9 (*a*) or α-p30 sera (*b*). Lectin-specific activities (determined by an HA assay and expressed as HA titer/mg of protein) of material pooled in V_o, V_i1, and V_i2 peaks were 0, 94,117, and 66,661, respectively.
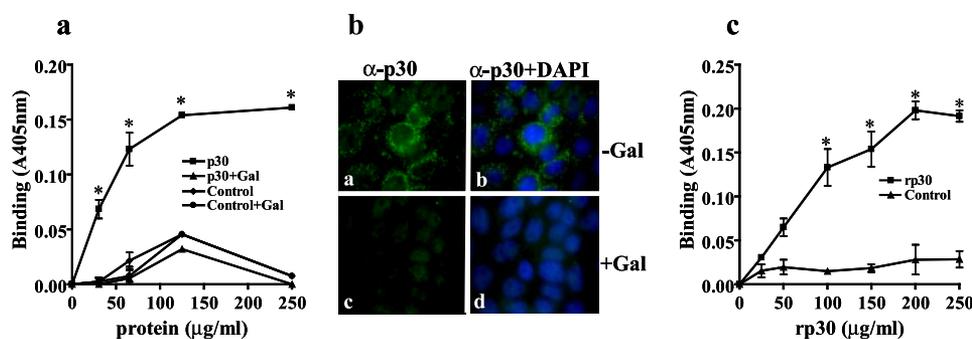
FIGURE 9. **Gal-inhibitable binding of p30 to Caco-2A cells.** *a,* Caco-2A cells were incubated with serial doubling dilutions of soluble phase proteins or control (β-galactosidase) in the presence or absence of 1 M Gal, and binding of p30 was detected by ELISA using α-rp30 sera. *, $p < 0.05$ for binding of p30 compared with the control and for binding of p30 compared with p30 + Gal. *b,* Caco-2A cells were incubated with p30 in the absence (*panels a* and *b*) or presence (*panels c* and *d*) of 1 M Gal, and binding was detected by IFA using α-rp30 sera. *DAPI,* 4′,6-diamidino-2-phenylindole. *c,* Caco-2A cells were incubated with serial doubling dilutions of rp30 or control (a recombinant protein containing the fusion tags), and binding of rp30 was detected by ELISA using α-rp30 sera. $p < 0.05$ for binding of rp30 compared with the control. Results of representative experiments are shown.

TABLE 4

Carbohydrate binding specificity of rp30

Saccharides	IC$_{50}$[a] (mM)	RIC[b]
Gal	12.5	1
GalNAc	6.5	2
Glc	>500	0.025
GlcNAc	>500	0.025
Man	>500	0.025
Fuc	>500	0.025
p-Nitrophenyl-α-Gal	25	0.5
p-Nitrophenyl-β-Gal	50	0.25
Me-α-Glc	>500	<0.025
Gal-(β1–3)-GalNAc	3.5	3.5
Gal-(α1–4)-Gal	2.5	5
Gal-(β1–3)-GlcNAc	1.5	8
Gal-(β1–6)-GlcNAc	0.5	25
Gal-(β1–4)-Glc	5	2.5
Fructose-(β1–2)-Glc	>500	<0.025

[a] Carbohydrate concentration that is required to inhibit 50% binding of rp30 to Caco-2A cells.
[b] Relative inhibitory capacity compared to Gal.

the binding of p30 to Caco-2A cells was mediated by the Gal binding activity of the protein.

Similarly, purified rp30 also bound to fixed Caco-2A cells in a dose-dependent and saturable manner (Fig. 9*c*). The carbohydrate specificity of rp30 binding was determined using a panel of mono- and disaccharides. Consistent with the carbohydrate specificity of the Gal affinity-purified material, GalNAc and Gal were the most potent monosaccharide inhibitors of binding of rp30 to Caco-2A cells, whereas Gal and GalNAc containing disaccharides were 2.5–25 times more inhibitory than Gal (Table 4).

rp30 Blocks Sporozoite Attachment and rp30 and α-rp30 Sera Inhibit C. parvum Infection of Intestinal Epithelial Cells—To determine whether adherence of sporozoites to host cells is mediated by p30, we used an ELISA-based attachment assay. This indicated that rp30 significantly inhibited sporozoite adherence to intestinal epithelial Caco-2A cells in a dose-dependent manner when compared with a control recombinant protein containing only the fusion tags (Fig. 10*a*).

To determine whether inhibition of sporozoite adherence by p30 resulted in a decrease in *C. parvum* infection of host cells, we used an ELISA-based infection assay. Preincubation of host cells with rp30 resulted in a significant reduction of infection compared with preincubation with the same concentrations of

the control protein (Fig. 10*b*). Similarly, preincubation of oocysts with α-rp30 sera resulted in statistically significant inhibition of infection compared with nonimmune sera (Fig. 10*c*). Taken together, these findings suggest that p30 binds to host cells and mediates attachment to and infection of host cells.

DISCUSSION

Carbohydrate-binding proteins or lectins have been identified in a number of parasitic protozoa and are implicated in mediating their attachment to host cells. Several previous studies have suggested that Gal/GalNAc-binding protein(s) on the parasite and/or host cells may be involved in attachment of *Cryptosporidium* sp. to intestinal epithelial cells. However, the identity of the putative Gal/GalNac-binding protein(s) has not been reported previously. In this study we identified p30, a secreted, Gal/GalNAc-specific lectin from *C. parvum*, which associates with the mucin-like microneme glycoprotein gp900 through protein-carbohydrate interactions, localizes to the apical region of sporozoites, binds to intestinal epithelial cells, and mediates *C. parvum* attachment to and infection of these cells *in vitro*. Native p30 exists as a homodimer or homotetramer, is encoded by a single copy gene that is conserved between both major species of *Cryptosporidium*, and is expressed within 24–72 h following *C. parvum* infection of intestinal epithelial cells.

Among apicomplexan parasites, most proteins believed to be important in attachment, invasion, and parasitophorus vacuole formation are present on the surface or are secreted from apical complex organelles such as micronemes, rhoptries, and dense granules (36, 37). These proteins are exocytosed or "shed" during gliding motility and subsequent invasion of host cells. Although the ultrastructural localization of p30 remains to be determined, the finding that it is present in the apical region of sporozoites and in shed or exocytosed material suggests that it is present in apical secretory organelles, consistent with its proposed role in attachment to host cells.

The Gal affinity-purified material containing gp900, gp40/15, gp40, and p30 had a high lectin-specific activity that was most specific for Gal and GalNAc. gp900 and gp40, which could be separated from p30 by gel filtration in the presence of Gal, showed no HA activity after extensive dialysis to remove Gal suggesting that these proteins are not responsible for the lectin activity in the affinity-purified material. However, both dimeric and tetrameric forms of p30 displayed high HA titers indicating that this protein is a Gal-binding lectin. This was confirmed by the specific binding activity of rp30 to Gal-agarose. The recombinant protein, however, did not display HA activity indicating that unlike the native protein it does not form dimers or tetramers.

Co-purification of p30 with gp900 and gp40 by Gal-affinity chromatography and identification of p30 as a Gal-bind-
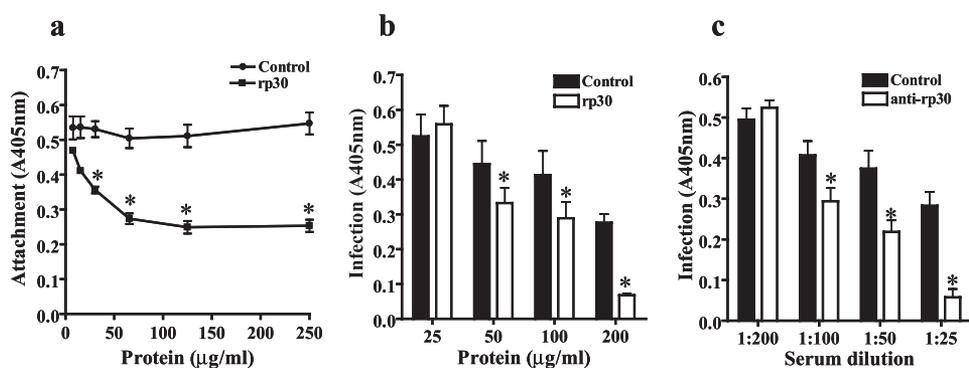
FIGURE 10. **rp30 blocks sporozoite attachment and rp30 and α-rp30 inhibit *C. parvum* infection of Caco-2A cells.** *a,* fixed Caco-2A cells were preincubated with rp30 or control protein followed by incubation with *C. parvum* sporozoites and sporozoite attachment quantified by ELISA. *, $p < 0.05$ compared with control protein. *b,* Caco-2A cells were preincubated with rp30 or control protein, followed by incubation with oocysts for 24 h, and infection was quantified by ELISA. *, $p < 0.05$ compared with control protein. *c, C. parvum* oocysts were preincubated with α-p30 sera or nonimmune mouse sera, followed by incubation with Caco-2A cells for 24 h and infection quantified by ELISA. *, $p < 0.05$ compared with nonimmune sera. Results of representative experiments are shown.

ing lectin suggests an association of p30 with these glycoproteins. gp900 is a >900-kDa mucin-like, membrane-bound, microneme glycoprotein that is exocytosed or shed from the surface of sporozoites during excystation and gliding motility (33). gp900 binds to intestinal epithelial cells (38) and the purified native protein as well as a recombinant cysteine-rich domain (domain 3), and antibodies to this domain all inhibit *C. parvum* infection of host cells *in vitro*, implicating gp900 in mediating attachment and/or invasion (33). gp40/15 is a mucin-like glycoprotein that is synthesized as a precursor protein that is proteolytically cleaved into gp40 and gp15 (31, 39). gp15 is GPI-anchored in the sporozoite membrane (34), whereas gp40, which is a secreted protein, is localized to the surface membrane by association with gp15 (35). gp40 binds to intestinal epithelial cells, and antibodies to it inhibit infection *in vitro*, suggesting that this protein also mediates attachment and invasion (31). Both gp900 and gp40 contain *O*-linked Gal and GalNAc residues (20). The finding that p30 could be separated from these mucin-like glycoproteins in the presence of an excess of Gal suggests that the association between them may be mediated by lectin-carbohydrate interactions.

Gal or Gal/GalNAc-specific lectins have been identified in a number of protozoan parasites. The best characterized of these is the Gal/GalNAc-specific lectin of the intestinal parasite *Entameba histolytica* (40). This cell surface-associated protein consists of a heavy subunit and GPI-anchored light and intermediate subunits, each encoded by different genes, and mediates binding to host cells, cytotoxicity, complement resistance, encystation, and cyst wall formation. *Trypanosoma cruzi* also expresses a 67-kDa surface-associated Gal-binding protein, which is implicated in mediating attachment to host cells (41). Among the apicomplexan protozoan parasites, *Sarcosystis muris* merozoites express a Gal-binding protein that is present in micronemes and contains two Apple domains (42). The *Toxoplasma gondii* micronemal adhesin Mic 1, which contains two TSP-1 domains, is also a Gal-binding protein (43). NcMIC4, a *Neospora caninum* lactose-binding protein, is present in micronemes, contains six Apple domains, and is implicated in binding to cell surface glycosaminoglycans (44).

Cryptosporidium p30 is not predicted to contain potentially adhesive domains such as Apple, epidermal growth factor, or TSP-1 domains nor does it have significant homology with mammalian C-type lectins or galectins. However, this may not be surprising, because the Gal/GalNAc-specific lectin of *E. histolytica*, which has been convincingly shown to mediate adherence, does not contain these domains and also lacks sequence identity to other well known lectins (45).

p30 has a signal sequence but does not have a predicted transmembrane domain or GPI anchor site, indicating that it is a soluble, secreted protein. This is confirmed by the findings that it is present in the soluble phase following Triton X-114 phase separation and is also present in proteins exocytosed from sporozoites during excystation.

Although gp900 does have a transmembrane domain (33), it is also shed from sporozoites during excystation (20) and gliding motility (33). This is consistent with the finding that the majority of gp900 is present in the soluble phase following Triton X-114 phase separation. It is likely, as is known to occur with other apicomplexan membrane-bound microneme proteins (46, 47), that gp900 is released from the membrane as a result of proteolytic cleavage. gp40, which partitions into the detergent phase by association with the GPI-anchored gp15, is also shed from the surface of sporozoites by an unknown mechanism (35).

Because p30 associates with gp900 and gp40, all of which are implicated in mediating attachment, these proteins may form a functional adhesive complex. This would be similar to the related apicomplexan parasite *T. gondii,* in which microneme proteins are thought to function as adhesive complexes consisting of a membrane-bound "escorter" protein that ensures correct micronemal targeting and one or more soluble adhesins. For example, the membrane-bound micronemal protein TgMIC 6 functions as an escorter for the soluble adhesive proteins TgMIC 1 and TgMIC 4 (47–50). TgMIC 1, which forms the core of the complex, simultaneously interacts with TgMIC 4 and TgMIC 6 as well as with host cells, thus forming a "bridge" between parasite and host cells (48, 49). In addition to functioning as an escorter, TgMIC 6 is also thought to have adhesive functions (49). Similarly, it is possible that p30, gp900, and gp40 function as an adhesive complex. Because p30 is a multivalent Gal/GalNAc-specific lectin that associates with gp900 and gp40, both of which contains Gal/GalNAc residues, this protein may be involved in bridging the parasite and host cell during the invasion process. However, further studies are needed to define the role of p30, gp900, and gp40 in *Cryptosporidium*-host cell interactions and to determine whether they can serve as interventional targets for cryptosporidiosis.

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