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Characterization of an Intestinal Epithelial Cell Receptor Recognized by the *Cryptosporidium parvum* Sporozoite Ligand CSL

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The protozoan parasite *Cryptosporidium parvum* is a leading cause of diarrhea in humans and neonatal calves. The absence of approved parasite-specific drugs, vaccines, and immunotherapies for cryptosporidiosis relates in part to limited knowledge on the pathogenesis of zoite attachment and invasion. We recently reported that the *C. parvum* apical complex glycoprotein CSL contains a zoite ligand for intestinal epithelial cells which is defined by monoclonal antibody (MAb) 3E2. In the present study, the host cell receptor for CSL was characterized. For these studies, a panel of epithelial and mesenchymal cell lines was examined for permissiveness to *C. parvum* and the ability to bind CSL. Cells of epithelial origin were significantly more permissive and bound significantly greater quantities of CSL than cells of mesenchymal origin. Caco-2 intestinal cells were selected from the epithelial panel for further characterization of the CSL receptor. Immunoelectron microscopy demonstrated that CSL bound initially to the surface of Caco-2 cells and was rapidly internalized. The molecule bound by CSL was identified as an 85-kDa Caco-2 cell surface protein by radioimmunoprecipitation and CSL affinity chromatography. Sporozoite incubation with the isolated 85-kDa protein reduced binding of MAb 3E2. Further, attachment and invasion were significantly inhibited when sporozoites were incubated with the 85-kDa protein prior to inoculation onto Caco-2 cells. These observations indicate that the 85-kDa protein functions as a Caco-2 cell receptor for CSL. CSL also bound specifically to intestinal epithelium from calves, indicating receptor expression in a second important host species. Molecular characterization of the CSL receptor may lead to novel avenues for disrupting ligand-receptor interactions in the pathogenesis of *C. parvum* infection.

The apicomplexan parasite *Cryptosporidium parvum* is an important cause of diarrhea in humans and in calves and other economically important food animals worldwide (27). Although significant advances have occurred (3, 16, 17, 21, 25, 33, 43, 47, 62, 65–68, 79, 80, 84, 86), prevention and treatment of the disease remain problematic due to the absence of approved vaccines or immunotherapies and the lack of consistently effective parasite-specific pharmaceuticals (reviewed in references 4, 13, 21, 72, 90, and 95). The refractory nature of persistent *C. parvum* infection to existing therapies may relate to the parasite's autoinfective life cycle stages, superficial compartmentalization within the host cell, and novel metabolic pathways (19, 27, 90). Of additional fundamental significance, limited knowledge on the pathogenesis of attachment of the infective zoite stages to host cells and subsequent invasion has hampered development of targeted intervention strategies for cryptosporidiosis.

Because apical complex and surface molecules of *C. parvum* (3, 16–18, 39, 43–45, 47, 52, 62, 66, 67, 73–75, 77, 79, 80, 85) and other closely related apicomplexan parasites (7, 8, 11, 14, 15, 29, 35, 36, 41, 51, 60, 64, 83, 87, 88) are involved in attachment, invasion, and intracellular development (2, 5, 8, 20, 23, 61, 63, 68, 84), such molecules may provide rational targets for immunological or pharmacological therapy. Additionally, the host cell receptors to which such parasite molecules bind may provide novel avenues for receptor-based control strategies (12, 14, 15, 30, 57, 62, 64, 82). Clearly, disruption of zoite attachment and invasion events essential to the life cycle of *C. parvum* would prevent initiation of primary infection or allow termination of existing infection. To this end, our studies have focused on CSL, an ~1,300-kDa conserved apical complex glycoprotein expressed by the infective sporozoite and merozoite stages of *C. parvum* (47, 73, 77, 80). CSL was originally identified by a monoclonal antibody (MAb), designated 3E2, which prevents sporozoite attachment and invasion in vitro and passively protects against *C. parvum* infection in vivo (77, 80). Consistent with these observations, we subsequently determined that CSL contains a zoite ligand which is involved in attachment to intestinal epithelial cells during the infection process (47). In brief, CSL was shown to bind specifically to intestinal epithelial cells in a dose-dependent, saturable, and self-displaceable manner and, once bound, to significantly diminish their permissiveness to infection by *C. parvum* sporozoites (47). In the present study, the host receptor recognized by CSL was characterized. We hypothesized that the receptor would be surface exposed and conserved on cells of epithelial origin. Here we report that cells of epithelial origin are significantly more permissive to *C. parvum* than mesenchymal cells, paralleling their ability to bind CSL. Using the Caco-2 human intestinal epithelial cell line (69), binding of CSL was localized

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to the microvillar surface, after which rapid internalization occurred. Sporozoite attachment, as well as binding of MAb 3E2, was inhibited after sporozoite incubation with an affinity-purified 85-kDa Caco-2 cell surface protein specifically recognized by CSL but not after incubation with mesenchymal cell control protein from rat endothelium. In further support of the biological relevance of these observations, CSL also bound to mucosal epithelium from the natural site of infection in neonatal calves, an agriculturally important host species for *C. parvum*. Collectively, these findings indicate that epithelial cells permissive to *C. parvum* express a receptor for CSL. Such parasite ligand-host receptor interactions may present exploitable events for novel molecular approaches in the control of cryptosporidiosis.

MATERIALS AND METHODS

Oocyst and sporozoite isolation. The Iowa *C. parvum* isolate (40) used in all experiments was maintained in newborn *Cryptosporidium*-free Holstein calves (76). Oocysts were isolated by density gradient centrifugation (1), stored in 2.5% (wt/vol) KCr_2O_7 (4°C), and used within 30 days of collection. Oocysts were hypochlorite treated immediately prior to excystation (76). Sporozoites were isolated by passage of the excysted preparation through a polycarbonate filter (2.0-μm pore size) and used immediately (47).

Evaluation of epithelial and mesenchymal cell permissiveness to *C. parvum* and ability to bind CSL. The epithelial lineage cell lines Caco-2 (human colonic adenocarcinoma; ATCC HTB37) (69), Intestine 407 (human embryonic jejunoileum; recently designated HeLa human cervical adenocarcinoma cell positive by the American Type Culture Collection; ATCC CCL6) (42), IEC-6 (rat small intestinal crypt; ATCC CRL1592) (71), MDBK (bovine kidney tubule; ATCC CCL22) (54), and HCT-8 (human ileocecal adenocarcinoma; ATCC CCL244) (89) were compared to the mesenchymal lineage cell lines VERO 76 (African green monkey kidney fibroblast; ATCC CRL1587) (70), BHK-21 (baby hamster kidney fibroblast; ATCC CCL10) (53), and RSE-1 (rat liver sinusoidal endothelium) (96) for permissiveness to *C. parvum* in three replicate experiments. Briefly, each cell line was grown to ~90% confluency in complete minimal essential medium (MEM) (MEM containing 10% fetal bovine serum, 1% nonessential amino acids, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) on replicate ($n = 6$) glass coverslips in 24-well plates and then uniformly inoculated with isolated sporozoites (1.2×10^5 in 200 μl of MEM per coverslip) as previously described (47). Following incubation (2 h, 37°C), the inoculation medium was aspirated and replaced with 1 ml of complete MEM. At 24 h postinoculation (p.i.), three replicate coverslip monolayer cultures for each cell line were washed with phosphate-buffered saline (PBS), methanol fixed, and processed for immunofluorescence assay (IFA) using *C. parvum*-specific MAb 4B10 and affinity-purified fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), IgM, and IgA to detect intracellular stages (47). Each coverslip culture was systematically examined in entirety by the same investigator using epifluorescence microscopy to determine the mean number of *C. parvum* stages per monolayer (47). The remaining three replicate coverslip cultures for each cell line were trypsinized, and individual cells were counted to determine the mean number of cells comprising an infected monolayer. Because the number of cells per monolayer varied significantly among cell lines, the mean numbers of intracellular stages were corrected for the number of cells per monolayer to determine the mean percentage of infected cells for each cell line [(mean number of intracellular stages per monolayer ÷ mean number of cells per monolayer) × 100].

To assess the ability to specifically bind CSL, epithelial and mesenchymal cell monolayers were cultured to ~90% confluency in 96-well plates and then incubated (30 min, 4°C) in triplicate with native ^{125}I-CSL (0.5 μg in 30 μl of PBS per monolayer) which had been isolated by isoelectric focusing (IEF) and radioiodinated as previously described (47). Monolayers were then centrifuged to remove incubation medium, washed extensively with PBS (4°C), and assayed in a gamma counter to determine bound ^{125}I-CSL counts per minute. Nonspecific binding of ^{125}I-CSL to each cell line was determined as previously described to calculate specific binding (47, 48). After determination of the mean number of cells comprising a monolayer for each cell line (as described above), the counts per minute were corrected to calculate the mean specifically bound counts per minute per 10^3 cells. The mean numbers of intracellular stages and mean counts per minute of ^{125}I-CSL bound for each cell line were then examined for significant differences using Student's one-tailed t test. All significance conclusions were verified in replicate experiments.

Immunoelectron microscopic localization of specifically bound CSL. To identify the site of CSL binding, Caco-2 cells were cultured to ~90% confluency in 16-well chamber slides and then incubated (2 to 6 min, 21°C) with IEF-isolated CSL (2.0 μg in 50 μl of PBS per monolayer). Cells were then washed extensively with PBS, fixed (2% [vol/vol] paraformaldehyde, 0.5% [vol/vol] glutaraldehyde, 15 min, 21°C), and processed for immunoelectron microscopy (6, 73). Sections were blocked and incubated sequentially with either MAb 3E2 or isotype-matched control MAb of irrelevant specificity (each at 22 μg/ml), affinity-purified goat anti-mouse IgM (Zymed, San Francisco, Calif.), and affinity-purified colloidal gold-conjugated rabbit anti-goat IgG (Zymed), with washing after each incubation (77). Sections were then postfixed, stained with uranyl acetate and Reynolds lead citrate, and observed with a JEOL 100 CX transmission electron microscope at 80 kV (73).

Evaluation of the ability of calf intestinal epithelium to bind CSL. Segments of terminal ileum from a *Cryptosporidium*-free 10-day-old Holstein bull calf were collected, placed in OCT compound (Miles Laboratories, Elkhart, Ind.), and snap frozen in liquid nitrogen as previously described (97). Samples were sectioned, fixed in acetone, rehydrated in a series of ethanol solutions, washed, and blocked (30 min, 37°C) with PBS containing 3.2% (wt/vol) fish gelatin and 2% (wt/vol) bovine serum albumin (BSA). Replicate sections were then incubated (30 min, 37°C) with IEF-isolated CSL or the negative control glycoproteins CPC205 or Tf190 (7.5 μg in 250 μl of PBS each). CPC205, a 205-kDa *C. parvum* oocyst wall glycoprotein defined by MAb 4D3, was isolated from oocyst shells by continuous elution gel electrophoresis for use in these studies as previously described (47). Tf190, an ~190-kDa *Tritrichomonas foetus* adhesion glycoprotein complex defined by MAb 32.3B3.5 (10, 81), was isolated from whole *T. foetus* by preparative electrophoresis as previously described (47). Following incubation, sections were washed extensively with PBS, processed for IFA, and counterstained with 0.1% (wt/vol) Evan's blue (47). Binding specificity was assessed by epifluorescence microscopy using MAb 3E2 for sections incubated with CSL, MAb 4D3 for sections incubated with CPC205, or MAb 32.3B3.5 for sections incubated with Tf190 and fluoresceinated goat anti-mouse IgG, IgM, and IgA. In parallel, replicate sections of each sample were processed identically using isotype-matched control MAbs of irrelevant specificity. IFA observations were validated in three replicate experiments.

Radioimmunoprecipitation and determination of M_rs of Caco-2 cell proteins recognized by CSL. Surface proteins of Caco-2 cells and RSE-1 control cells were radioiodinated by the lactoperoxidase technique as previously described (32, 74). In brief, 3×10^7 Caco-2 or RSE-1 cells (each 92% viable by trypan blue dye exclusion) were washed with PBS and then suspended in PBS (750 μl) containing lactoperoxidase (0.03 mg) and ^{125}I-labeled Na (0.5 mCi), to which H_2O_2 was subsequently added. Following incubation (4 min, 21°C), the reaction was terminated by washing the cells with PBS containing 1 mM KI (4°C). Radiolabeled cells were then solubilized in lysis buffer containing protease inhibitors (Sigma, St. Louis, Mo.) [50 mM Tris (pH 8.0), 5 mM 4-(2-aminoethyl)benzene-sulfonylfluoride hydrochloride, 0.3 μM aprotinin, 10 μM E-64, 0.01 mM leupeptin, 5 mM EDTA, 130 μM bestatin, and 0.5% (vol/vol) Triton X-100], centrifuged ($50,000 \times g$, 30 min, 4°C) to remove insoluble material, and stored at −80°C prior to use. MAb 3E2 and an isotype-matched control MAb of irrelevant specificity, each derived from serum-free culture supernatant, were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma) according to the manufacturer's protocol. In brief, each MAb (4 mg in 1 ml of buffer [0.1 M $NaHCO_3$, 0.5 M NaCl]) was ultracentrifuged ($100,000 \times g$, 30 min, 4°C) to remove insoluble material and then gently mixed (4 h, 4°C) with CNBr-activated Sepharose 4B (0.8 ml). After ~90% coupling, the reaction was terminated by incubation (1 h, 4°C) with 1 M ethanolamine-HCl (32). MAb-Sepharose preparations were then washed with PBS (4°C) and used immediately for immunoprecipitation as described below.

Radiolabeled Caco-2 and RSE-1 cell surface protein preparations were precleared of nonspecifically binding molecules by incubation (2 h, 4°C) with CNBr-activated Sepharose and then isotype-matched control MAb-coupled Sepharose. Precleared samples (the amount derived from the soluble fraction of 10^7 cells each) were incubated (30 min, 4°C) on a rocking platform with either IEF-isolated CSL (8.0 μg in 350 μl of PBS) or PBS (350 μl) and then mixed (1.5 h, 4°C) with either MAb 3E2-Sepharose or isotype-matched control MAb–Sepharose (each containing 100 μg of MAb). MAb-Sepharose preparations were then collected and washed three times with TSA buffer (0.01 M Tris-HCl [pH 8.0], 0.5 M NaCl, 0.5% [vol/vol] Triton-X 100), three times with TSA buffer containing 0.1% (wt/vol) bovine hemoglobin, and three times with 0.05 M Tris-HCl (pH 6.8). The preparations were then boiled (5 min, 100°C) in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer

(47) and ultracentrifuged. The soluble fractions containing immunoprecipitates were collected and electrophoresed in 10 to 20% and 2 to 12% gradient SDS-polyacrylamide gels. Radiolabeled Caco-2 and RSE-1 cell surface proteins (4.7×10^4 cpm for each cell line) were processed for SDS-PAGE identically and included for comparison. Autoradiography was performed as previously described (32).

CSL affinity chromatography isolation of Caco-2 cell proteins. IEF-purified CSL (100 μg) was coupled to NHS-Sepharose (1-ml HiTrap column; Amersham Biotech, Piscataway, N.J.) according to the manufacturer's protocol, washed (50 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.5% [wt/vol] octylglucoside), and preeluted prior to use. After determination of optimal conditions for binding and elution, preparative isolation of Caco-2 molecules bound by CSL was performed as follows (4°C, all steps). Caco-2 cells (1.7×10^6) were solubilized in binding buffer (PBS containing 2% [wt/vol] octylglucoside and protease inhibitors, pH 7.5), centrifuged to remove the insoluble fraction, and then bound to CSL-coupled NHS-Sepharose. After washing (50 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.5% [wt/vol] ocytlglucoside; 20 column volumes), specifically bound material was eluted (50 mM diethylamine [pH 11], 0.05% [wt/vol] octylglucoside; 10 column volumes), immediately neutralized (0.1 M Tris-HCl, pH 6.8), and then dialyzed (3.5-kDa exclusion limit) against PBS and stored at −80°C prior to use. The concentration of eluted protein was determined by micro-bicinchoninic acid assay (Pierce, Rockford, Ill.). The number and molecular weights of eluted protein species were determined by 2 to 12% and 10 to 20% gradient SDS-PAGE and silver staining (77). To monitor for possible leaching of CSL from the column, eluate (1 μg) was evaluated for immunoreactivity with MAb 3E2 by Western blotting using previously described methods (77). To further evaluate the specificity of binding of Caco-2 molecules by CSL during affinity chromatography, the column eluate was examined by dot immunoblot assay as follows (77). Replicate nitrocellulose membranes were dotted with eluate (5 μg per dot), fixed, blocked with buffer A containing 3.2% (wt/vol) fish gelatin and 2% (wt/vol) BSA, and incubated (30 min, 21°C) with CSL, CPC205, or Tf190 (2 μg in 200 μl of PBS each). After washing, membranes were incubated (30 min, 21°C) with MAb 3E2 for CSL detection, MAb 4D3 for CPC205 detection, MAb 32.3B3.5 for Tf190 detection, or isotype-matched control MAbs of irrelevant specificity (each MAb at 25 μg/ml), washed, and then incubated with affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, and IgA followed by substrate.

Functional evaluation of affinity-isolated Caco-2 cell proteins. To further assess the biological activity of affinity-isolated Caco-2 proteins, their ability to bind surface-exposed CSL on sporozoites was evaluated. Viable sporozoites (8×10^3 per treatment) were incubated (10 min, 4°C) with affinity-isolated Caco-2 proteins (20 μg in 160 μl of MEM), the soluble fraction from either Caco-2 or RSE-1 whole-cell lysates (100 μg, each in 230 μl of MEM), or MEM and then divided onto poly-L-lysine-coated multiwell glass slides and air dried. Whole-cell lysates for these experiments were prepared by solubilization in binding buffer as described above and then dialyzed (4°C, 12- to 14-kDa exclusion limit) against PBS prior to use. The slides were processed for IFA using MAb 3E2 or isotype-matched control MAb as described above and then evaluated by epifluorescence microscopy for inhibition of MAb 3E2 binding to sporozoites in two replicate experiments. Next, the effect of the cell preparations on sporozoite infectivity was determined in two replicate experiments. Viable sporozoites (6×10^4 per treatment) were incubated (15 min, 37°C) in triplicate with affinity-isolated Caco-2 proteins (5 μg in 300 μl of MEM), the soluble fraction from either Caco-2 or RSE-1 whole-cell lysates (5 or 250 μg, each in 300 μl of MEM), or MEM and then inoculated onto individual Caco-2 coverslip cultures. At 24 h p.i., cultures were processed for IFA using MAb 4B10 as described above to determine the total number of *C. parvum* stages per monolayer. The mean numbers of intracellular stages for each treatment group were examined for significant differences using Student's one-tailed *t* test. In parallel, sporozoite viability was determined after incubation under identical conditions with affinity-isolated Caco-2 proteins (5 μg in 300 μl of MEM) or the soluble fraction from each whole-cell lysate (250 μg in 300 μl of MEM) using fluorescein diacetate and epifluorescence microscopy (76). For each treatment group, a minimum of 200 sporozoites was observed to quantitate viability.

RESULTS

Permissiveness to *C. parvum* is highest in cells of epithelial lineage and parallels quantitative binding of CSL. Based on the percentage of cells infected at 24 h p.i., variation in permissiveness to *C. parvum* was observed in both epithelial (0.12 ± 0.05 to $0.35 \pm 0.08\%$) and mesenchymal (0.003 ± 0.001 to $0.062 \pm 0.014\%$) cell lines (Fig. 1). However, all epithelial cell lines were significantly more permissive than mesenchymal cell lines (Fig. 1) ($P < 0.025$ to $P < 0.00001$). The rationale for the experimental design in host cell permissiveness studies was as follows. Isolated sporozoites rather than oocysts were used so that the infective dose could be accurately quantitated and standardized, and the potential for error introduced by differences in oocyst excystation could be avoided. Use of isolated sporozoites also eliminated the potential for error in quantitating infection introduced by the presence of unexcysted oocysts. In the assay used, the gentle washing conditions required to prevent monolayer disruption may not remove all oocysts. Remaining adherent oocysts can be difficult to distinguish from intracellular stages (26, 46). Because of the nonuniform distribution of infection and focal development of *C. parvum* stages known to occur in vitro (91), the entire monolayer was examined for each cell line. Finally, to accurately calculate percent infection, the number of cells comprising a monolayer for each cell line was determined to correct for known variations in cell size and growth characteristics. Paralleling their higher permissiveness levels, Intestine 407, Caco-2, and MDBK epithelial cells bound significantly more CSL than each mesenchymal cell line (Fig. 1) ($P < 0.04$ to $P < 0.007$). IEC-6 and HCT-8 epithelial cells also bound more CSL than mesenchymal cell lines, but only the differences between IEC-6 and VERO-76 or RSE-1 cells were significant (Fig. 1) ($P < 0.05$). Considered collectively, the mean counts per minute of CSL bound by all epithelial cell lines was significantly greater than that bound by all mesenchymal cell lines ($P < 0.0009$). To extend the biological relevance of these observations to an agriculturally important host species for *C. parvum*, the ability of CSL to bind intestinal epithelial cells from the natural site of infection in neonatal calves was examined. CSL, but not the control glycoproteins CPC205 and Tf190, bound selectively to villous and crypt epithelial cells based on immunofluorescence reactivity (Fig. 2).

CSL binds to the host cell surface and is rapidly internalized. For CSL binding localization and receptor characterization, Caco-2 cells were selected as a representative epithelial cell line because of their human intestinal origin, permissiveness to *C. parvum* and corresponding ability to bind CSL, and widespread use in studies on the biology of *C. parvum* infection (9, 16–18, 24, 26, 33, 34, 37, 44, 49, 55, 65, 90–92), including those in which the CSL ligand was identified and characterized (47). Immunoelectron microscopy localized the initial binding of CSL to a surface-exposed receptor on Caco-2 cells, hereafter referred to as CSL-R (Fig. 3). Focally dense labeling was frequently observed. Internalization rapidly followed binding, with both events being observed within 2 min of cell exposure to CSL (Fig. 3A). Internalization was progressive as evidenced by an increase in the density of intracellular labeling after 6 min of incubation with CSL (Fig. 3B). To further evaluate whether CSL internalization was an active receptor-mediated process, the experiment was repeated at 4°C. Because monolayers began to detach when incubated at 4°C, morphologic artifacts that precluded ultrastructural evaluation were introduced during subsequent washing and fixation steps.

CSL-R is an 85-kDa cell surface protein. The M_r of CSL-R was determined as part of its initial characterization. An 85-kDa protein in surface-radioiodinated Caco-2 cells was bound

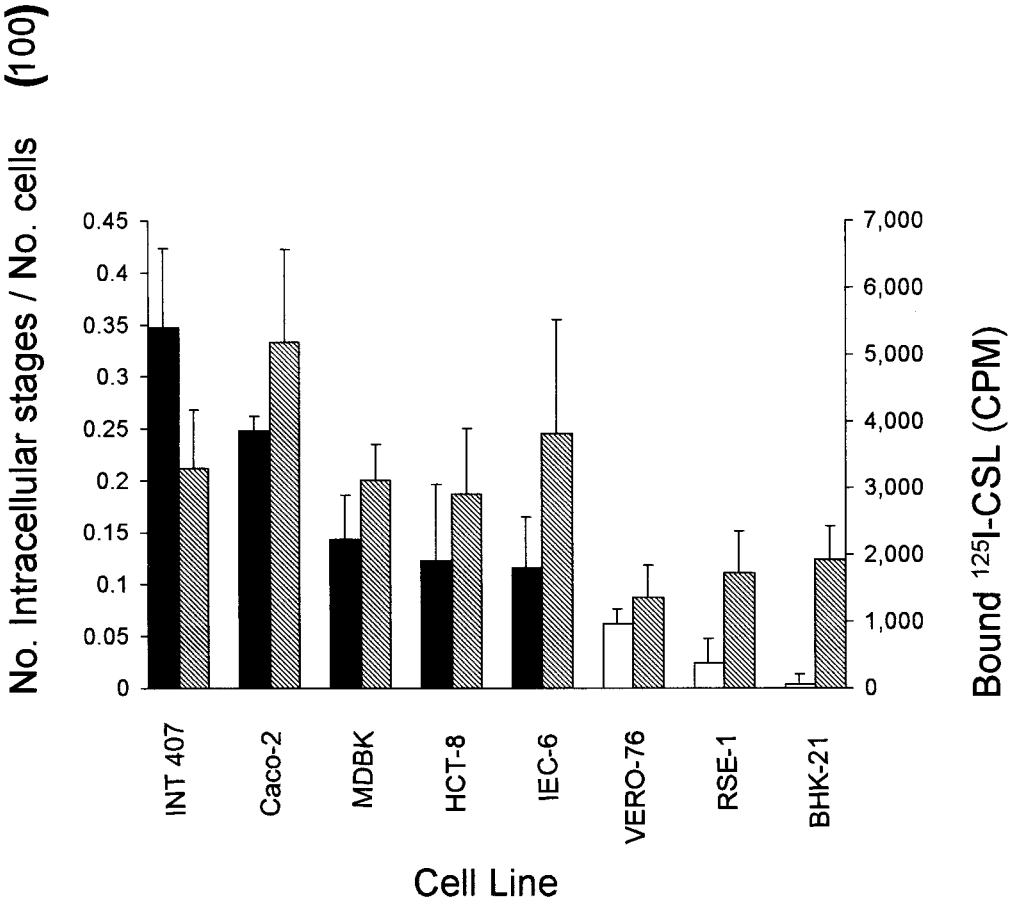

FIG. 1. Comparative permissiveness of epithelial (■) and mesenchymal (□) cell lines to infection with *C. parvum* sporozoites and ability to bind CSL (▨). Error bars indicate standard deviations.

by CSL and immunoprecipitated by MAb 3E2-Sepharose (Fig. 4, lanes 1 and 2). In contrast, no radioiodinated surface proteins were bound by CSL and immunoprecipitated from the minimally permissive RSE-1 mesenchymal cell line (Fig. 4, lanes 4 and 5). To corroborate these findings, Caco-2 cell lysates were next examined by CSL affinity chromatography. Because MAb 3E2 recognizes a repetitive carbohydrate-dependent epitope in CSL (77) thought to be important in binding of CSL-R (47), NHS-Sepharose was used to optimize exposure of carbohydrate moieties in coupled CSL. Consistent with radioimmunoprecipitation results, an 85-kDa protein was isolated from Caco-2 cells by CSL affinity chromatography (Fig. 5). The 85-kDa protein was authenticated as being Caco-2 cell derived, and not a degradation product of column-bound CSL, based on the absence of MAb 3E2 reactivity with affinity column eluate in Western blots. In addition, CSL, but not CPC205 or Tf190, bound to the 85-kDa Caco-2 protein following its isolation, as demonstrated by immunoblots using MAbs specific for each glycoprotein (Fig. 6). Collectively, these findings confirmed that binding between CSL and CSL-R is specific and that CSL-R is preferentially expressed by epithelial cells permissive to *C. parvum*.

CSL-R binds to *C. parvum* sporozoites and inhibits attachment and invasion. Incubation of sporozoites with affinity-isolated CSL-R or Caco-2 whole-cell lysate substantially reduced binding of MAb 3E2, based on a marked diminution of immunofluorescence reactivity. In contrast, strong immunofluorescence reactivity of MAb 3E2, indistinguishable from that reported previously (77), was observed with sporozoites after incubation with RSE-1 whole-cell lysate or MEM. These observations provided indirect evidence that CSL-R bound to a ligand moiety in CSL recognized by MAb 3E2 or that bound CSL-R sterically hindered MAb 3E2 binding. Based on these observations, the effect of CSL-R binding on infectivity was determined. The infectivity of sporozoites incubated with CSL-R prior to inoculation onto Caco-2 monolayers was significantly reduced compared to that of sporozoites incubated with Caco-2 or RSE-1 whole-cell lysates (Table 1). Sporozoite infectivity following incubation with Caco-2 cell lysate was also significantly reduced compared to that of sporozoites incubated with RSE-1 cell lysate (Table 1). Further, this reduction was dose dependent, being significantly greater at the higher concentration of Caco-2 lysate evaluated (Table 1). The infectivity of sporozoites incubated with RSE-1 cell lysate, compared to medium, was significantly reduced; however, there was no significant difference in infectivity between sporozoites incubated with RSE-1 lysate at either concentration evaluated (Table 1). Viability of sporozoites was not significantly affected by any of the treatments examined (MEM, 96%; CSL-R, 90%; Caco-2 cell lysate, 95%; RSE-1 cell lysate, 92%).

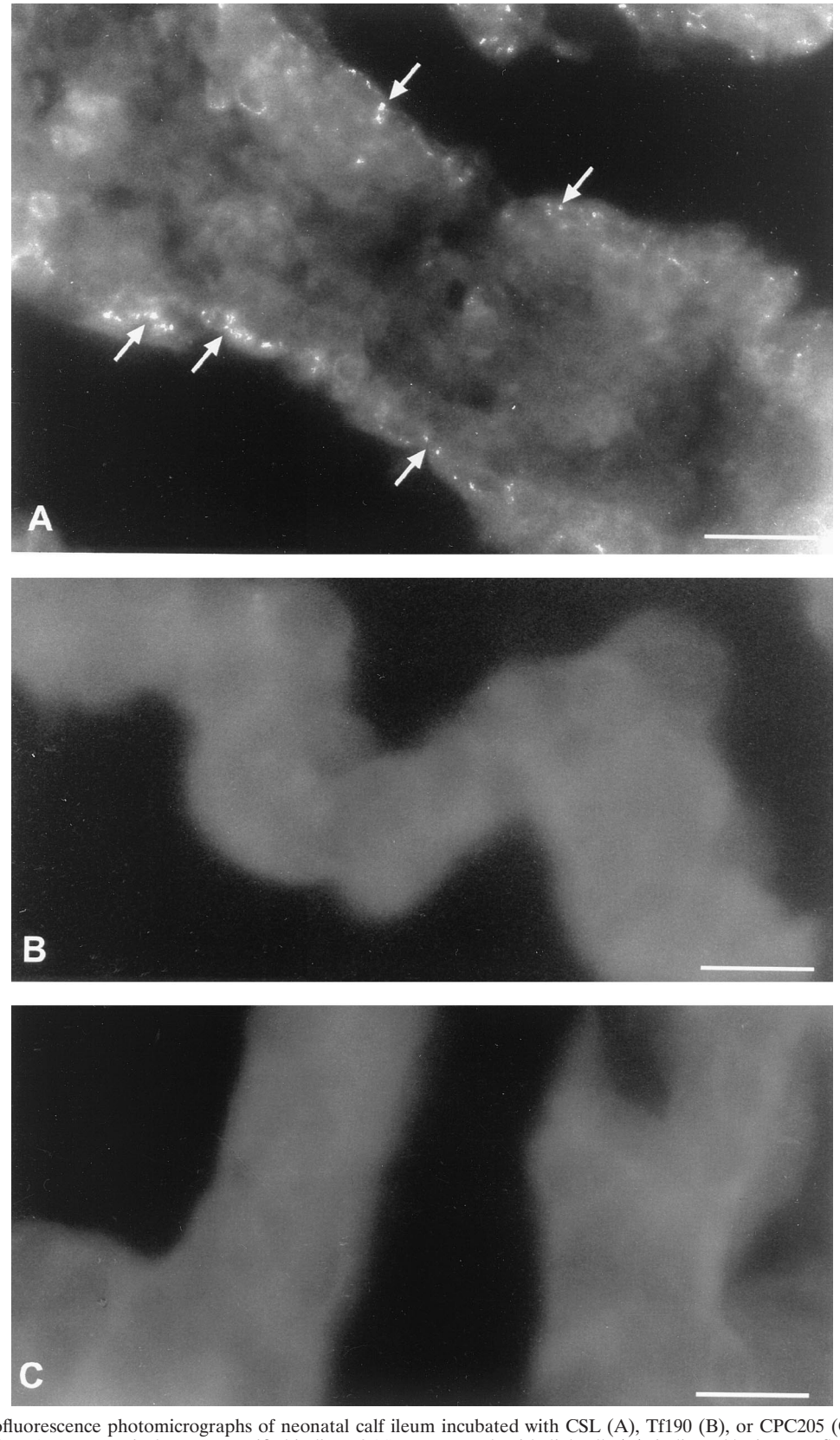

FIG. 2. Immunofluorescence photomicrographs of neonatal calf ileum incubated with CSL (A), Tf190 (B), or CPC205 (C) and probed with MAb 3E2, 32.3B3.5, or 4D3, respectively. Note specific binding of CSL to mucosal epithelial cells (A), indicated by immunofluorescence reactivity (arrows), and absence of binding of the control glycoproteins Tf190 (B) and CPC205 (C). Bars, 5 μm.

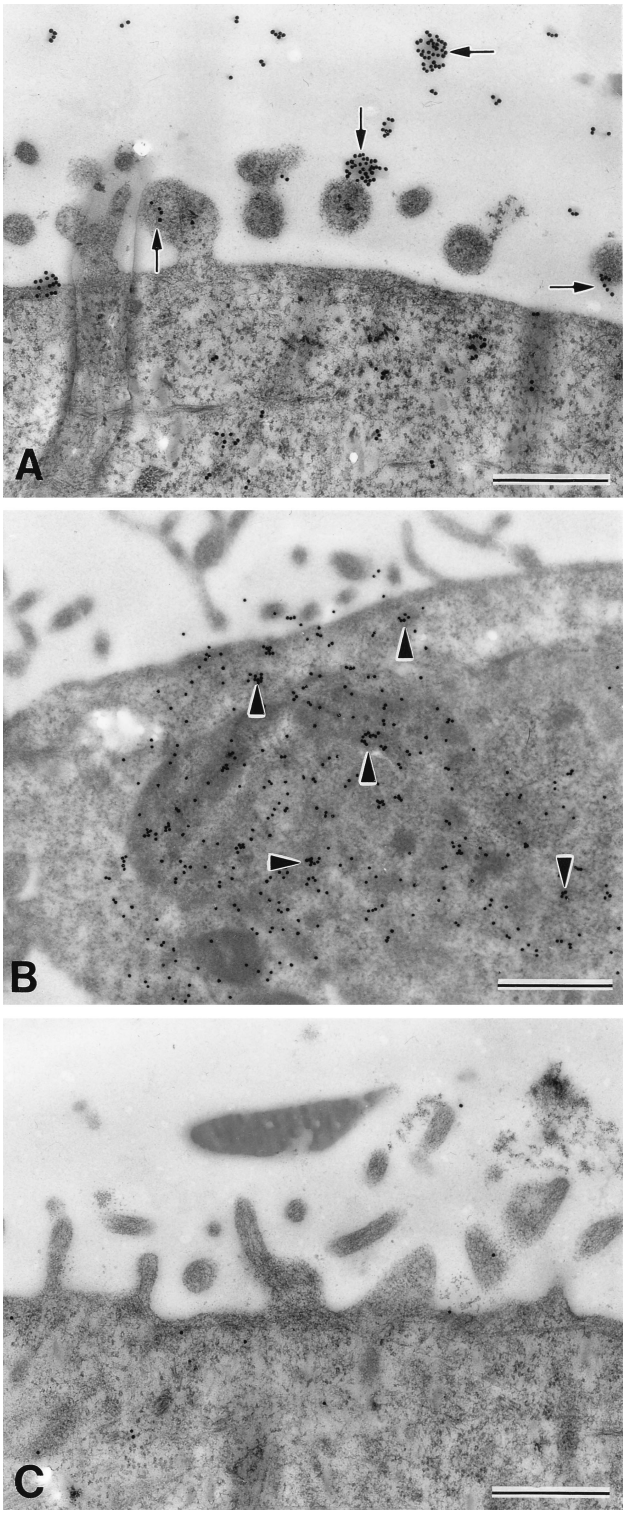

FIG. 3. Immunoelectron photomicrographs of Caco-2 cells incubated for 2 min (A) or 6 min (B and C) with CSL and then probed with MAb 3E2 (A and B) or isotype-matched control MAb (C). Note dense multifocal immunogold labeling of surface microvilli (arrows) initially (A), followed by progressively increased intracellular labeling (arrowheads) over time (B). Bars, 1 μm.

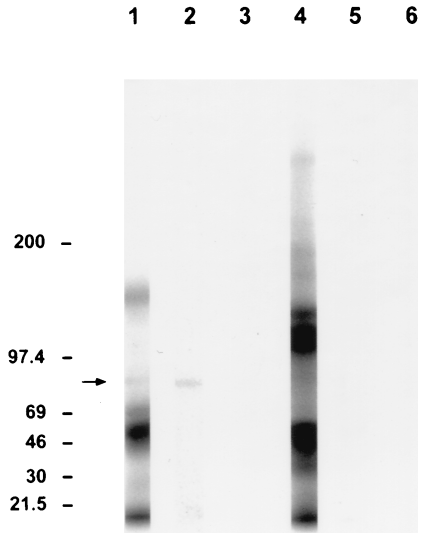

FIG. 4. SDS-PAGE gel autoradiograph demonstrating an 85-kDa surface protein (arrow) in ^{125}I-labeled Caco-2 cells (lane 1) bound by CSL and immunoprecipitated by MAb 3E2 (lane 2). ^{125}I-labeled RSE-1 cell surface proteins before (lane 4) and after (lane 5) incubation with CSL and precipitation with MAb 3E2 are shown for comparison. Lanes 3 and 6 were loaded with isotype control MAb-CSL precipitates from ^{125}I-labeled Caco-2 or RSE-1 cells, respectively. Molecular mass standards are indicated on the left in kilodaltons (myosin, 200 kDa; β-galactosidase, 97.4 kDa; BSA, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and trypsin inhibitor, 21.5 kDa) (Amersham Pharmacia Biotech).

DISCUSSION

Attachment and invasion mechanisms mediated by the interaction of specific parasite and host molecules have been described for apicomplexan protozoa closely related to *C. parvum*, including *Toxoplasma gondii* (5, 11, 12, 29, 35, 36, 60, 64), *Neospora caninum* (41, 51, 83), *Eimeria tenella* (2, 7, 87, 88), and *Plasmodium* spp. (14, 15, 20, 30, 57, 82). However, there have been relatively few reports of either putative or defined parasite ligand-host receptor interactions in *C. parvum*, and these have all been recent (3, 16–18, 44, 47, 62, 84, 85). The high efficiency of *C. parvum* infection, indicated by low infective doses, and the rapidity with which sporozoites locate, attach to, and invade host cells first suggested that a zoite ligand-host receptor relationship may mediate infection in cryptosporidiosis (27, 52, 72, 91). Studies to investigate this possibility resulted in the recent identification of the CSL ligand (47, 77). Our continuing studies have been directed towards an improved understanding of the biology of parasite-host interactions in cryptosporidiosis, as such information is essential to targeted drug discovery, immunization, and other specific modalities for life cycle disruption (72, 75, 80). Thus, in the present study the host cell receptor for CSL, designated CSL-R, was characterized.

A wide variety of epithelial (9, 16, 22, 28, 31, 38, 45, 59, 78, 86, 92–94, 98, 99) and mesenchymal (50, 56, 58, 78, 92) cell lines have been shown to support *C. parvum* growth in vitro and have been used in model systems to study the biology of parasite development and the efficacy of candidate anticryptosporidial agents (reviewed in references 90 and 91). It is difficult to make accurate quantitative comparisons between the

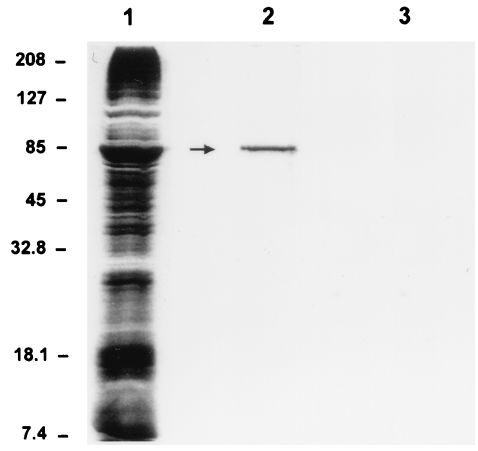

FIG. 5. Silver-stained SDS-polyacrylamide gel demonstrating an 85-kDa protein (arrow) in Caco-2 cells (lane 1, 4.0 μg) isolated by CSL affinity chromatography (lane 2, 0.3 μg). Lane 3 was loaded with sample buffer to identify silver stain artifacts. Molecular mass standards (Bio-Rad, Hercules, Calif.) are indicated on the left in kilodaltons (myosin, 208 kDa; β-galactosidase, 127 kDa; BSA, 85 kDa; carbonic anhydrase, 45 kDa; soybean trypsin inhibitor, 32.8 kDa; lysozyme, 18.1 kDa; and aprotinin, 7.4 kDa).

epithelial and mesenchymal cell lines examined in those studies and the present study for the ability to support *C. parvum* growth due to differences in the experimental designs. Confounding variables include the isolate, life cycle stage and method used for culture inoculation, time of incubation prior to quantitation of infection, type of fixation, method used to identify and quantitate stages, and, perhaps of most significance, formulae by which infection levels were calculated and data presented. Among the cell lines examined in the present study, quantitative infection levels, corrected for the number of cells comprising a monolayer, indicated that cells of epithelial origin are significantly more permissive to *C. parvum* than cells of mesenchymal origin. These findings are consistent with the report by Upton et al. (91, 92), and anecdotal observations by others, that most epithelial or epithelial-like cell lines optimally support *C. parvum* growth in vitro.

The hypothesis that cells of epithelial lineage would express a receptor for CSL was based on the observed in vivo tropism of *C. parvum* for intestinal epithelium, the natural site of infection in mammals, as well as for extraintestinal mucosal epithelium (27). Indeed, the ability of *C. parvum* to infect a wide variety of epithelia in vivo, including those of intestinal,

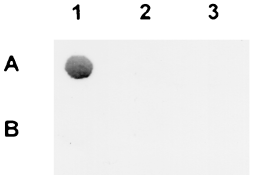

FIG. 6. Dot immunoblot demonstrating specific binding of CSL to CSL-R following isolation from Caco-2 cells. Shown are affinity-purified CSL-R dotted onto nitrocellulose and incubated with CSL (lane 1), Tf190 (lane 2), or CPC205 (lane 3); and probed with MAbs specific for each glycoprotein (row A) or isotype-matched control MAbs (row B).

TABLE 1. Effect of Caco-2 and RSE-1 cell proteins on *C. parvum* infectivity in vitro

Sporozoite treatment (μg)	No. of intracellular Stages (mean ± SD) in expt:	
	1	2
MEM	789 ± 15	881 ± 18
CSL-R (5)	211 ± 8[a,b]	258 ± 10[a,b]
Caco-2 cell lysate (5)	495 ± 27[a,c]	517 ± 17[a,c]
Caco-2 cell lysate (250)	326 ± 23[a,c,d]	337 ± 27[a,c,d]
RSE-1 cell lysate (5)	644 ± 14[a]	700 ± 13[a]
RSE-1 cell lysate (250)	627 ± 22[a]	717 ± 19[a]

[a] Significantly lower than the value for MEM-treated sporozoites ($P < 0.005$).
[b] Significantly lower than the values for Caco-2 lysate ($P < 0.01$, 5 or 250 μg)- or RSE-1 lysate ($P < 0.005$, 5 or 250 μg)-treated sporozoites.
[c] Significantly lower than the values for RSE-1 lysate-treated sporozoites ($P < 0.005$, 5 or 250 μg).
[d] Significantly lower than the value for sporozoites treated with 5 μg of Caco-2 lysate ($P < 0.005$).

biliary, pancreatic, respiratory, endometrial, and conjunctival origin (27), suggested the existence of one or more conserved receptors for parasite ligands such as CSL. In addition, previous ultrastructural studies have demonstrated that initial attachment of *C. parvum* follows site-specific orientation of the anterior pole of zoites to the luminal surface of intestinal epithelial cells and binding to microvilli (27). In support of the receptor hypothesis, we observed specific binding of radiolabeled CSL to epithelial cell lines, selective binding of CSL to mucosal epithelium in calf intestinal tissue, and ultrastructural localization of bound CSL to the surface and microvilli of Caco-2 cells. The multifocally dense labeling pattern observed ultrastructurally suggests that CSL-R has a nonuniform distribution or, alternatively, that coalescence and aggregation of CSL-R follows complexing with CSL. This observation and the progressive increase in apical intracellular labeling over time suggest that binding between CSL-R and CSL may trigger internalization. Such a possibility is consistent with the observation that zoites are enveloped by microvilli following attachment to enterocytes and subsequently are internalized within a superficial parasitophorous vacuole during infection (27). Further supporting the receptor hypothesis, and extending our previous findings that CSL contains a ligand defined by MAb 3E2, we observed that sporozoite incubation with affinity-purified CSL-R reduced binding of MAb 3E2. Paralleling this observation, the ability of sporozoites to attach and invade was reduced in a dose-dependent manner by incubation with CSL-R, independent of any nonspecific effect on sporozoite viability. These findings indicate specific binding between CSL-R and CSL on the sporozoite surface and are consistent with a moderately high-affinity interaction ($K_D = 2.74 \times 10^{-9}$ M) as observed in previous studies on CSL binding kinetics (47). While incubation with RSE-1 cell lysate also reduced sporozoite infectivity, compared to incubation with medium, there was no reduction compared to incubation with Caco-2 cell lysate or isolated CSL-R. This observation and the absence of a dose-dependent effect of RSE-1 lysate on infectivity suggest that the reduction was due to undefined, nonspecific factors. Finally, we conclude that CSL-R is an 85-kDa cell surface protein based on its isolation from Caco-2 cells by both CSL affinity chromatography and immunoprecipitation, specific rec-

ognition by CSL in immunoblots of the affinity-purified Caco-2 cell preparation, and absence of an 85-kDa protein in 3E2-CSL immunoprecipitates from RSE-1 cells.

Although infection levels and binding of radiolabeled CSL were significantly higher in epithelial cells than in mesenchymal cells, the two parameters were not uniformly parallel. While this observation is consistent with CSL-R expression in epithelial cells, the following additional possibilities are suggested. Mesenchymal cells may express CSL-R but in low copy number or variant structure, either of which could account for lower CSL binding and sporozoite entry. Ligand-receptor interactions in addition to CSL–CSL-R could also be operative during infection in the cell lines examined. Several *C. parvum* (glyco)proteins have recently been reported to have a defined or putative role in sporozoite binding and entry into host epithelial cells, including GP900 (3), CP47 (62), gp15/40 (16, 17, 85), Gal/GalNAc lectin (18, 39, 44), and thrombospondin-related anonymous protein C1 (84). Of these, CP47 was reported to bind a 57-kDa surface protein on HCT-8 human ileal adenocarcinoma cells (62); host cell receptors for other *C. parvum* (glyco)proteins have not yet been reported. These studies (3, 16–18, 39, 44, 62, 84, 85) and the present report suggest that *C. parvum* zoites may have redundant mechanisms for infection of different host cell types or may use more than one ligand in a multistep process, each having functional domains which differ biochemically and in binding specificity. The glycosylated state of CSL and carbohydrate dependency of the epitope recognized by MAb 3E2 suggest carbohydrate-mediated binding to CSL-R. However, peptide domains in CSL or glycosylated active sites in CSL-R could also be involved in specific recognition. Consistent with the preceding possibilities, previous kinetics studies suggested that CSL binding to Caco-2 cells occurs with true or apparent negative cooperativity (47). Such binding may result from steric effects related to multivalent receptors or ligands or to multiple receptor or ligand subpopulations (48). Finally, the possibility that non-ligand-receptor-mediated entry mechanisms may exist and serve a redundant role during infection cannot be excluded.

In summary, the results presented here indicate that a surface-exposed epithelial cell receptor is specifically bound by the CSL ligand and that the ability of cells to bind CSL parallels their permissiveness to *C. parvum* in vitro. These findings provide a biological basis for the observation that epithelial cells are preferentially infected in vivo and are significantly more permissive than mesenchymal cells in vitro. The observation that infectious sporozoites have a reduced ability to attach and invade after incubation with isolated CSL-R and that this effect is likely due to blockade of CSL further supports specificity of binding. Ongoing molecular studies may provide insight into additional modalities targeting CSL-R, CSL, or both to structurally or functionally disrupt ligand-receptor interactions involved in the pathogenesis of *C. parvum* infection.

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