Identification and Initial Characterization of Five Cryptosporidium parvum Sporozoite Antigen Genes

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Cryptosporidium parvum, an Apicomplexan parasite of gastrointestinal epithelial cells, causes severe disease in persons with AIDS and is a common cause of self-limited diarrhea in children, animal handlers, and residents of developing countries. No approved therapy exists; in research studies, however, hyperimmune bovine colostrum raised to Cryptosporidium oocysts and sporozoites has eradicated disease or decreased parasite burden in some AIDS patients. Although the protective antigens recognized by bovine hyperimmune colostrum have not been defined, protective antigens of other Apicomplexan parasites frequently have been associated with two unique structures of invasive forms, the trilaminar pellicle and the apical complex. In order to identify immunogenic Cryptosporidium proteins that may be protective antigens for use as recombinant immunogens in passive and/or active immunotherapy, we screened two genomic DNA expression libraries with polyspecific anti-Cryptosporidium antibodies. We used an approach to cloning apical complex and pellicle protein antigens that succeeded despite the lack of large numbers of organisms that would be necessitated for conventional biochemical approaches requiring organelle or membrane purification. We report here the molecular cloning of five C. parvum genes and the characterization of the cognate sporozoite proteins having molecular masses of >500, 68/95, 45, 23, and 15/35 kDa. The light microscopic immunofluorescence pattern of antibodies recognizing these protein antigens suggests that they are located in the pellicle or apical complex of Cryptosporidium sporozoites.

Cryptosporidium parvum, a parasitic agent of acute, self-limited diarrhea in immunocompetent hosts (35), causes a more severe and potentially lethal disease in persons with AIDS (7, 21, 26). In spite of the morbidity of cryptosporidiosis in persons with AIDS, no effective immunotherapy or chemotherapy is available and our understanding of the biology and biochemistry of C. parvum, as well as of the pathophysiology of cryptosporidiosis, is at an early stage. C. parvum has been classified on the basis of ultrastructural features as a member of the phylum Apicomplexa. Invasive stages of members of the Apicomplexa ("zoites" = sporozoites, merozoites, and tachyzoites) are extracellular and have a unique trilaminar membrane, the pellicle, which appears to mediate attachment to the host cell membrane and movement into the parasitophorous vacuole and is associated with subpellicular structures involved in motility. Zoites also share an anterior apical complex composed of specialized secretory organelles: rhoptries, micronemes, and dense granules. These organelles secrete products which appear to have roles in rendering the host cell membrane penetrable by the zoite and in generating the parasitophorous vacuole. Cryptosporidium infection is initiated by ingestion of oocysts, excystation of oocysts with release of sporozoites, and invasion of gut epithelial cells by sporozoites. Maturation of merozoites intracellularly, release of merozoites, and reinvasion of gut epithelial cells follow. The Cryptosporidium sexual cycle also occurs in the gut, resulting in production of sporulated oocysts, some of which may excyst before being shed. In persistent infection of the immunocompromised host, both the merozoite and the endogenously produced sporozoite are postulated to contribute to ongoing invasion but the relative contribution of each stage has not been determined (11).

Sporozoites and merozoites are the only infective stages which are free in the gut and accessible to neutralization by luminal antibody. An extensive body of literature indicates that pellicle and apical complex proteins of other Apicomplexan zoites, e.g., of Plasmodium, Eimeria, and Toxoplasma spp., are targets of invasion-inhibiting antibody in vitro and neutralization in vivo (22–25, 34). Thus, proteins localized in these structures of Cryptosporidium zoites are also expected to be targets of protective immune responses. Other investigators have described several surface antigens of Cryptosporidium sporozoites and merozoites that appear to be targets of neutralizing antibodies (1, 3, 4, 15, 16, 18, 23, 29, 30). Anti-Cryptosporidium hyperimmune bovine colostral immunoglobulin reacts with numerous oocyst and sporozoite proteins on Western blots (immunoblots) (28) and significantly protects against sporozoite challenge in animals, although the relevant protective antigens have not yet been characterized (9). Similar animal protection results have been reported for whole hyperimmune bovine colostrum (1, 8, 10, 18). Furthermore, duodenal infusion of hyperimmune bovine colostrum has been reported to ameliorate or eradicate Cryptosporidium infection in AIDS or other immunocompromised patients (31–33). Taken together, these observations indicate that passive immunotherapy of cryptosporidiosis is feasible in AIDS and other immunocompromised patients and that active immunotherapy of more immunologically intact hosts may also limit or prevent disease manifestations.

We describe here the identification and characterization of five groups of lambda gt11 recombinant clones encoding immunogenic Cryptosporidium proteins that appear to reside in the sporozoite pellicle or apical complex and that, therefore, are candidate protective antigens. We used an ap-

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approach to cloning apical complex and pellicle protein antigens that avoided the conventional biochemical approach of organelle or membrane purification requiring large numbers of oocysts. The endogenous sporozoite antigens encoded by the cloned genes have molecular masses of >500, 68/95, 45, 23, and 15/35 kDa, and the light microscopic indirect immunofluorescent antibody (IFA) patterns of polyclonal antibody affinity-purified on each fusion protein suggests that the corresponding endogenous protein is located in the sporozoite apical complex or pellicle.

MATERIALS AND METHODS

Parasites. Cryptosporidium sp. oocysts isolated from San Francisco General Hospital patients with AIDS were used in the production of polyclonal antibodies. Cryptosporidium sp. oocysts from calves (Bruce Anderson, University of Idaho, Caldwell) were used for the isolation of DNA for construction of lambda gt11 genomic expression libraries. C. parvum oocysts of the AUCP-1 isolate (Byron Blagburn, Auburn University, Auburn, Ala.) propagated in Holstein calves were used for Western blots and IFA studies. Oocysts were isolated and purified from feces as described previously (12).

Preparation of anti-oocyst/sporozoite antibodies. Female BALB/c mice at 10 weeks of age were immunized four times intraperitoneally with approximately 5 × 10⁵ sonicated oocysts. Polyclonal antibodies in mouse ascites reacted with the sporozoite surface, with the oocyst surface, and with internal antigens by IFA.

Screening of Cryptosporidium DNA expression libraries and preparation of REAs. Two Cryptosporidium lambda gt11 genomic expression libraries were constructed with DNA from approximately 2.5 × 10⁴ to 5 × 10⁵ sporozoites per library. A restriction fragment expression library has been previously described (12, 17). A second expression library was constructed by using aliquots of Cryptosporidium DNA which were partially digested with various concentrations of DNase I in 33 mM Tris-HCl (pH 7.4)–5 mM CaCl₂ for 15 min. EDTA was added to 20 mM, and the aliquots were extracted once with phenol and combined. The pooled sample was extracted twice with phenol, twice with chloroform-isooamyl alcohol, and once with diethyl ether before ethanol precipitation. The DNA was subsequently treated with Klenow and T4 DNA polymerases to prepare staggered ends and with EcoRI methylase to protect internal EcoRI sites and ligated to EcoRI linkers (pCCGAAATTCGG). Linkers were removed by EcoRI digestion followed by exclusion chromatography on Sephadex G-100. DNA eluting in the void volume was ethanol precipitated and ligated to EcoRI-cleaved, alkaline phosphatase-treated lambda gt11 arms, packaged in vitro, and plated and amplified on Escherichia coli Y1090. The resulting library was 70% recombinant and contained 1.2 million independent clones. The two libraries were screened with polyclonal anti-Cryptosporidium oocyst/sporozoite antibodies, and positive clones were plaque purified. Antibodies, designated recombinant eluted antibodies (REAs), were affinity purified from polyclonal anti-Cryptosporidium oocyst/sporozoite antibodies on isopropyl-β-D-thiogalactopyranoside (IPTG)-induced confluent plaque lifts of purified lambda gt11 clones and eluted with 10 mM glycine (pH 2.6)–150 mM NaCl as previously described (2, 6, 19). Control REA was affinity purified from the same antibodies on wild-type lambda gt11 clones.

Sibling analysis of recombinant clones. Approximately 400 PFU of each of the lambda gt11 purified clones and wild-type lambda gt11 were individually dotted in a grid pattern onto a lawn of Y1090 on 152-mm petri dishes and incubated for 3 h at 37°C prior to application of a nitrocellulose filter saturated with 50 mM IPTG. After overnight incubation, filters were removed and washed three times in TBSTA (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween, 0.2% Na azide) and blocked with 1% bovine serum albumin-TBSTA. Each of 57 filters was incubated with a single REA prepared from one of the clones for 2 h, washed three times in TBSTA, and incubated with goat anti-mouse immunoglobulin G (IgG) conjugated to alkaline phosphatase (Promega) for 1 h prior to development with the colorimetric reagents nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. This methodology allowed us to place each of the 57 recombinants into sibling groups based on the immunologic cross-reactivity of their encoded antigens.

Localization of endogenous antigens in sporozoites and oocysts by indirect immunofluorescence. Slides containing air-dried, acetone-fixed sporozoites and oocysts were incubated with REAs, including control REA, for 1 h in a humidified chamber, washed with phosphate-buffered saline (pH 7.4), and incubated with affinity-purified goat anti-mouse IgG-IgA-IgM fluorescein isothiocyanate (Zymed). Slides were counterstained with Evan’s blue, coverslipped, and observed and photographed with a Nikon Optiphot microscope equipped for fluorescein immunofluorescence.

Identification of the endogenous antigen encoded by cloned gene fragments. One hundred million oocysts were lysed by five freeze-thaw cycles after suspension in 500 μl of a protease inhibitor cocktail containing 100 μM leupeptin (Sigma), 100 μM chymostatin (Sigma), 100 μM pepstatin (Sigma), 100 μM trans-e-poxysuccinyl-l-leucylamido(4-guanidino)butane (Sigma), 100 μM phenylmethylsulfonyl fluoride (Sigma), 50 mM N-α-para-tosyl-l-lysine chloromethyl ketone (Sigma), 150 mM NaCl, and 500 mM EDTA (pH 8.0). Western blots were prepared as previously described (20) and incubated with the individual REAs. Alkaline phosphatase-labeled second antibodies and substrates, as described above, were used to detect the REA-bound antigens. The Western blot shown in Fig. 3 was scanned with a Hewlett-Packard flatbed scanning densitometer in reflectance mode prior to photography. Nebulin (900 kDa) (27) and titin (2,500 kDa) (13) were used as high-molecular-mass standards and were gifts from Kuan Wang, University of Texas, Austin.

RESULTS

Use of REAs to identify immunogenic Cryptosporidium proteins and their cognate genes. In order to identify immunogenic Cryptosporidium proteins, we screened DNA expression libraries with polyclonal anti-Cryptosporidium antibodies known to react with sporozoites and oocysts. Two hundred seventy-five thousand plaques from the amplified restriction fragment library were screened; 52 clones expressing fusion proteins were identified and purified. Two hundred and twenty-five thousand plaques from the DNase library were screened; five positive clones were identified and purified. REAs were prepared from each of the 57 expressing clones and used as described below.

The use of REAs allows the identification and isolation of recombinants encoding immunogenic polypeptides and provides a reagent for further localization of these antigens to particular parasite structures or subcellular compartments by IFA or immunoelectron microscopy and for determining the Mₛ of the antigens by Western blot without the necessity
of producing antibodies to recombinant polypeptides in animals. This approach was valuable because it allowed us to identify 16 clones that express five distinct immunogenic Cryptosporidium proteins and to identify their cognate genes from a battery of 57 expressing clones, to localize these immunogenic proteins by IFA to the sporozoite pellicle and apical complex regions, and to determine the Ms of the endogenous sporozoite proteins. REAs from the other 41 expressing clones of the battery of 57 localized to oocysts, to other structures of the sporozoite, or could not be unequivocally characterized by IFA and Western blot and are not reported here.

The REA approach is complementary to a monoclonal antibody (MAb) approach, used by other Cryptosporidium researchers, for identifying a protein(s), localizing it to a particular structure or subcellular compartment, and investigating its biological effect. Although it requires a suitable expression library, the REA approach has several advantages. First, monospecific affinity-purified REA is, as opposed to any MAb, of polyclonal origin and therefore recognizes multiple protein epitopes, whereas MABs generated to Cryptosporidium antigens are often carbohydrate specific (25, 29, 30). Experimental limitations and difficulties often experienced with MABs that recognize carbohydrate epitopes or conformational epitopes are avoided by the REA approach. Second, very large amounts of recombinant protein can be produced to affinity purify REA or to make antibody in animals. Third, once REA recognizing the target of interest is identified, the corresponding gene, by definition, is in hand for further analysis.

Sibling grouping of recombinant clones by use of REAs. Sibling analysis of the 57 purified recombinants and wild-type lambda gti1 was performed using individual REAs. Recombinant clones were segregated into sibling groups expressing antigenically related or identical proteins on the basis of their cross-reactivity. For example, REA prepared from clone S34 identified the fusion proteins expressed by clones S34, S38, S41, and S57 but not the proteins expressed by the other 53 recombinants or wild-type lambda gti1 (Fig. 1). Correspondingly, REA from clone S38 identified clones S34, S38, S41, and S57 but none of the other clones. The number of clones in each of the sibling groups reported here is indicated in Table 1.

REA from five sibling groups recognized candidate sporozoite pellicle and apical complex proteins. In order to determine which of the sibling groups contained clones encoding Cryptosporidium proteins that reside in the sporozoite pellicle, we looked for the presence of a diffuse REA-IFA staining pattern of sporozoites. To identify proteins which appeared to be apical organelle proteins, we looked for REA-IFA staining of the anterior portion of sporozoites where rhoptries, micronemes, and dense granules are located.

REA from clones of the S34 group reacted with the anterior one-half of fixed sporozoites in IFA (Fig. 2A). REA from clones of the S19 group reacted with the anterior tip of the sporozoite in a very localized manner (Fig. 2B). REA from clones of the S2 group exhibited a flocculant pattern over sporozoites (Fig. 2C). REA from the single S7 clone reacted diffusely with the fixed sporozoite but not with the oocyst (data not shown). REA from the S24 group reacted diffusely with the fixed sporozoite in IFA (Fig. 2D). Control REA did not react with the sporozoite or the oocyst (data not shown).

Western blot analysis of REAs from the five sibling groups encoding sporozoite proteins. REAs from the five sibling groups encoding sporozoite proteins recognized distinct protein(s) on Western blots. REAs from clones of the S34 group identified a protein of >500 kDa on Western blots of oocyst/sporozoite proteins (Fig. 3). The protein migrated faster than titin at 2,500 kDa and slower than nebulin at 900 kDa (results not shown). REAs from clones of the S19 group reacted with a protein of 68 kDa and weakly recognized a protein of 95 kDa (Fig. 4, lane 2). S2 REA identified a 45-kDa protein (Fig. 4, lane 3). S7 REA detected a 23-kDa protein (Fig. 4, lane 4). REAs from the S24 group identified a 15-kDa protein doublet and a 32/35-kDa protein doublet on a Western blot (Fig. 4, lane 5).

Western blotting with murine oocyst/sporozoite antibodies from which the REAs were prepared (Fig. 4, lane 1) indicated that the antibodies reacted with many different oocyst/sporozoite antigens. Control REA prepared from wild-type lambda gti1 clones which express only β-galactosidase showed little reactivity with oocyst/sporozoite proteins (Fig. 4, lane 6), indicating that the elution process

![FIG. 1. Sibling analysis of Cryptosporidium lambda gti1 clones](http://iai.asm.org/)

**Table 1. Sporozoite proteins encoded by cloned genes of C. parvum**

<table>
<thead>
<tr>
<th>Sibling group</th>
<th>No. of clones</th>
<th>Fixed IFA pattern</th>
<th>Size (kDa) of endogenous antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>S34</td>
<td>4</td>
<td>1/2 sporozoite</td>
<td>&gt;500</td>
</tr>
<tr>
<td>S19</td>
<td>2</td>
<td>Apical</td>
<td>68/95</td>
</tr>
<tr>
<td>S2</td>
<td>4</td>
<td>Flocculant</td>
<td>45</td>
</tr>
<tr>
<td>S7</td>
<td>1</td>
<td>Diffuse</td>
<td>23</td>
</tr>
<tr>
<td>S24</td>
<td>5</td>
<td>Diffuse</td>
<td>15/35</td>
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yielded highly specific antibody. It is unclear whether the multiple proteins identified by the S19 and S24 REAs are due to antigenic cross-reactivity or posttranslational processing. Although we cannot completely rule out parasite proteolysis prior to lysis in protease inhibitors, it appears unlikely since use of the protease cocktail routinely allowed us to detect the >500-kDa protein of the S34 family in undegraded form, suggesting that protein degradation is minimal.

FIG. 2. IFA of fixed oocysts and sporozoites probed with REAs. (A) S34 REA; (B) S19 REA; (C) S2 REA; (D) S24 REA. REAs from members of the S34 and S19 groups recognized sporozoite apical antigens with dissimilar patterns in IFA. REAs from the S2 and S24 groups reacted in diffuse, but different, patterns with the whole sporozoite.

DISCUSSION

We have sought to identify the genes encoding immunogenic Cryptosporidium sporozoite proteins, particularly proteins which are localized in the pellicle and apical complex, with the intent of defining protective protein antigens and producing recombinant antigens in expression systems. The expression of recombinant antigens will allow the identifica-
tion of protective protein epitopes and, as C. parvum cannot be propagated in vitro, will solve the difficult or impossible task of isolating sufficient endogenous antigen from organisms propagated in calves for immunization purposes. Thus far, several Cryptosporidium proteins and glycoproteins reported to be the targets of neutralizing antibodies have been identified with MAbs (1, 4, 5, 16, 18, 23, 29, 30). However, these protective MAbs frequently recognize carbohydrate epitopes (23, 29, 30). Carbohydrate antigens, alone or in association with lipids or proteins, appear to be important in the immune response to C. parvum (5, 14). However, it would not be feasible to express carbohydrate-dependent epitopes of protective glycoproteins in prokaryotic expression systems which lack appropriate glycosylation enzymes. The approach we have taken to identifying protective antigens will allow us to avoid the identification of carbohydrate-dependent protective epitopes.

We report the identification of five Cryptosporidium genes that encode immunogenic sporozoite proteins which, by virtue of their IFA patterns, appear to be pellicle or apical complex proteins. Two of these, the 15/35- and 23-kDa proteins (1, 30), and possibly a third (23) are similar in size to protective antigens previously described; we do not yet know if they are the same. Further experiments, including assessment of the reactivity of these immunogenic proteins with protective hyperimmune bovine colostrum IgG and the ability of antibodies to the proteins, or the proteins themselves, to reverse or protect against sporozoite challenge in vitro and in vivo, will indicate whether we have cloned a protective antigen(s) suitable for use as an immunogen(s) in the development of passive or active immunotherapy for cryptosporidiosis.

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REFERENCES