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Isolation and expression of the gene for a major surface protein of *Giardia lamblia*

(protozoan parasite/attachment)

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ABSTRACT **To study the interactions between the parasitic protozoan *Giardia lamblia* and its environment, we have cloned the gene that encodes the two major surface-labeled trophozoite protein species. Sequence analysis of this gene reveals a single open reading frame specifying a hydrophilic, cysteine-rich (11.8%) protein of 72.5-kDa molecular mass with an amino-terminal signal peptide and a postulated hydrophobic membrane-spanning anchor region near the carboxyl terminus. Most of the cysteine residues (58 of 84) are in the motif Cys-Xaa-Xaa-Cys, which is dispersed 29 times throughout the sequence. Antibodies against the recombinant protein react with the entire surface of live trophozoites, including flagella and adhesive disc. These antibodies inhibit trophozoite attachment, prevent growth, and immunoprecipitate the major ≈66- and 85-kDa proteins from surface-labeled live trophozoites. The recombinant *Escherichia coli* also expresses polypeptides of ≈66- and 85-kDa molecular mass, which are not fusion proteins. This suggests that the processing and/or conformational changes that lead to production of these two peptide species in *E. coli* reflect those that occur in *Giardia*. The abundance of cysteine residues suggests that the native proteins on the parasite surface may contain numerous disulfide bonds, which would promote resistance to intestinal fluid proteases and to the detergent activity of bile salts and would help to explain the survival of *Giardia* in the human small intestine.**

Giardia lamblia is endemic and epidemic throughout the industrial and developing world (1). It is the major identified cause of waterborne enteric disease in the United States, where it is a particular problem in day-care centers and for wilderness hikers (1, 2). Its manifestations vary from debilitating severe diarrhea, malabsorption, and growth retardation to self-limited or even asymptomatic infection (1). Infection is caused by motile, flagellated *Giardia* trophozoites, which colonize the upper small intestine. This hostile environment harbors few other microorganisms (3) because it contains high concentrations of bile salts, degradative enzymes, and fluctuating levels of nutrients and hydrogen ions. This versatile protozoan has evolved mechanisms not only to survive this unfriendly environment but also to use components of its milieu for growth (4), attachment (5), and differentiation (6).

Attachment of *Giardia* trophozoites to the intestinal epithelium is crucial to both initial colonization and maintenance of infection, since parasites that do not attach or that actively "swim" against the flow of intestinal fluid would be expelled. Attachment may also damage the intestinal mucosa directly or cause malabsorption by affecting access of nutrients to the mucosal absorptive surface. Both biochemical and physical mechanisms of giardial attachment have been proposed. The former is based on observation of a trypsin-activated lectin (7) and the latter upon the creation of negative pressure between the trophozoite ventral adhesive disc and the substratum by beating of the ventral flagella and/or contraction of the rim of the disc (8). In either case, antibodies that adhere to the flagellar or disc surface may be expected to inhibit attachment.

Although parasites have evolved a variety of cell surface components as adaptations to survival in hostile environments, the structure and function of molecules on the surface of *G. lamblia* are largely unknown (19). To understand this host–parasite interface, we have cloned in *Escherichia coli*, expressed, and sequenced the entire gene (called *TSA 417*) that encodes the two major surface-labeled trophozoite antigen species.†† The importance of these trophozoite surface antigens (TSAs) is supported by our observations that rabbit antiserum against the recombinant protein reacts with the entire trophozoite surface, including the membrane that covers the flagella and adhesive disc. Moreover, this antiserum inhibits attachment and prevents growth of the parasite.

MATERIALS AND METHODS

Cultivation of *G. lamblia*. Trophozoites of *G. lamblia* strain WB (American type culture collection no. 30957) were grown to late logarithmic phase in TYI-S-33 medium (9) with bovine bile (10) but without added vitamins, iron, or antibiotics (6).

Construction and Screening of a Genomic DNA Library. *G. lamblia* DNA was partially digested with DNase I in the presence of 1 mM Mn^{2+} (11). Fragments of >1 kilobase (kb) were methylated with *Eco*RI methylase and repaired with Klenow DNA polymerase I. *Eco*RI linkers were added by blunt-end ligation, and the fragments were digested with *Eco*RI and ligated (12) into the *Eco*RI site of the expression vector Lambda ZAP (Stratagene; ref. 13). To screen for expression of *Giardia* membrane proteins, we used rabbit antiserum raised against a differential Triton X-114 detergent-phase extract of trophozoites prepared by the method of Bordier (14).

Determination of Nucleotide Sequence. The entire sequence of bases 1 to 2400 of the 3-kb D3 subclone (described below) was determined on both strands by the dideoxynucleotide method (15) with Sequenase 1.0 or 2.0 and ^{35}S-substituted dATP by using restriction nuclease deletions in the pBluescript plasmid or subclones in M13 mp18 or mp19 (16).

Abbreviation: TSA, trophozoite surface antigen.
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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. M33641).

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Expression of TSA 417 and Preparation of Antiserum Against the Recombinant Protein. *E. coli* XL1 carrying the *TSA 417* gene in the pBluescript plasmid (on the *D3* subclone) (Stratagene; ref. 13), was grown to early logarithmic phase. Half of the culture was induced to express the recombinant protein by incubation for ≈2 hr with 10 mM isopropyl thio-D-galactoside and half was used as an uninduced control. Since we have not purified recombinant TSA 417, for antigen preparation the induced *E. coli* was washed in phosphate-buffered saline (PBS), diluted to 70% transmission at 610 nm, sonicated for 3 min at full power, and boiled for ≈10 min. A rabbit was injected intravenously with 0.25 ml of this antigen on day 1, 0.5 ml on day 4, and 1.0 ml on days 6, 8, 11, and 13, and a booster was administered after 5 weeks; the rabbit was bled 10 days later (6). Anti-*E. coli* antiserum raised in the same way, by using induced *E. coli* XLI carrying the pBluescript plasmid with an unrelated insert, was used as control in each experiment.

Immunoblot Analysis of Recombinant Antigens. Proteins expressed by induced and control recombinant *E. coli* were solubilized in SDS sample buffer with 50 mM dithiothreitol, separated on SDS/PAGE (17), and transferred to nitrocellulose (18). Expressed *G. lamblia* proteins were identified by reactivity with the rabbit antiserum prepared against the *G. lamblia* Triton X-114 detergent-phase extract.

Immunoprecipitation of Labeled Trophozoite Proteins. Washed trophozoites were surface-labeled with ^{125}I by the Iodo-Gen method (19). For metabolic labeling with [^{35}S]-cysteine, attached trophozoites were washed with PBS and incubated for 4 hr at 37°C in Hepes/saline buffer with 10 mM ascorbic acid containing 2 mg of trypticase and 1 mg of yeast extract per ml (20). After extensive washing, they were solubilized in immunoprecipitation buffer C (21) containing 2 mM phenylmethylsulfonyl fluoride and were centrifuged for 5 min at 12,000 × *g* in the cold. The extracts were treated with anti-recombinant, anti-*E. coli*, or preimmune serum (1:10), and the immunocomplexes were isolated with protein A-agarose and then subjected to SDS/PAGE under reducing conditions. Gels were processed with EN^{3}HANCE (NEN) and dried, and the radiolabel was visualized by autoradiography.

Immunofluorescence Staining of Trophozoites with Anti-recombinant Antiserum. Trophozoites from late logarithmic-phase cultures were washed with PBS containing 10 mM ascorbic acid to promote parasite viability and were treated for 90 min at 4°C with heat-inactivated rabbit anti-recombinant TSA 417; controls were treated with preimmune serum from the same rabbit or the antiserum against *E. coli* with pBluescript (1:10). The parasites were washed by centrifugation and treated with a 1:800 dilution of fluorescein isothiocyanate-conjugated goat antiserum to rabbit immunoglobulins. Parasites remained motile and apparently viable throughout this procedure.

Frozen Section Immunoelectron Microscopy. Washed trophozoites were fixed with a mixture of 3% paraformaldehyde and 1% glutaraldehyde in PBS (pH 7.4) at room temperature for 1 hr. After a brief wash in PBS, the cells were pelleted, resuspended in 2.3 M sucrose, allowed to infuse for 10–20 min, and concentrated in a light pellet before the excess sucrose was removed. A 5-μl drop of the sucrose-infused cell suspension was then cryosectioned on a Porter–Blum MT-2B ultramicrotome equipped with a RMC FS-1000 cryostage (22). Sections (100 nm) were collected on carbon-coated copper grids. Sections were blocked with 2% gelatin for 10 min at room temperature and then incubated with 1:10 dilutions of either rabbit anti-recombinant TSA 417 or control preimmune serum. After a washing, sections were treated with 5-nm gold-conjugated goat anti-rabbit serum (1:10, Janssen Pharmaceutica). Adsorption staining with uranyl acetate and embedding in Carbowax–methyl cellulose were carried out as described (22). No immunogold was observed in sections treated with preimmune serum.

RESULTS

Identification of a Clone that Expresses the Major TSAs. To identify genes that encode major *G. lamblia* surface proteins, we used antiserum against a Triton X-114 detergent-phase extract of trophozoites, which agglutinates live trophozoites and immunoprecipitates most surface-iodinated trophozoite proteins, including the major proteins of molecular masses approximating 66 and 85 kDa. Since this antiserum reacts with multiple *G. lamblia* membrane antigens, we affinity-purified (23) the antibodies corresponding to each of 13 clones. SDS/PAGE and autoradiography showed that antibody purified against one clone, "417," immunoprecipitated the major surface-iodinated 66-kDa trophozoite antigen.

A pBluescript plasmid containing the *TSA 417* insert was excised from Lambda ZAP (13) for studies of the *Giardia* DNA insert and expression of the recombinant protein. Restriction enzyme analysis showed the *Giardia* DNA insert to be ≈7 kb, and deletion studies localized the *TSA 417* coding region to a 3-kb segment (called *D3*) proximal to the *lacZ* promoter (Figs. 1 and 2), which was used for all further studies. Western blots of isopropyl thio-D-galactoside-induced recombinant *E. coli* using the anti-*G. lamblia* Triton X-114 extract antiserum showed that parasite proteins of ≈66, 85, and 90 kDa were synthesized under the control of the plasmid *lacZ* promoter (Fig. 1) by both the original 7-kb insert and the 3-kb subclone. The 66- and 85-kDa proteins correspond to the surface-labeled TSAs immunoprecipitated by anti-recombinant antiserum (see below). We do not know the relationship of the 90-kDa band; however, it is a product of the *TSA 417* gene, since the 3-kb insert only has one long open reading frame.

Characterization of the Genomic *TSA 417* DNA and Deduced Amino Acid Sequences. The DNA and derived protein sequences of the *TSA 417* gene reveal several features that are of particular biological interest. The *D3* insert encoding TSA 417 has a single long open reading frame of 2139 base pairs (bp) (Fig. 2) preceded by stop codons in all three reading frames. Therefore, the expressed TSA 417 is not a fusion protein with the vector-encoded *lacZ* segment (Fig. 2). Moreover, this open reading frame has an ATG start signal 205

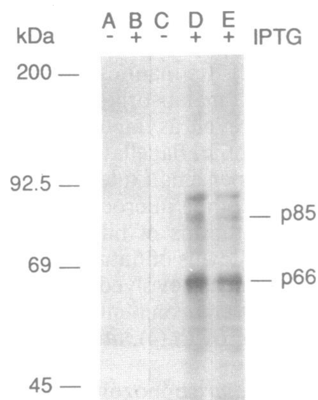

FIG. 1. Expression of *G. lamblia* proteins by recombinant *E. coli*. *E. coli XL1* carrying the pBluescript plasmid (13) with no insert (lanes A and B) or carrying the original ≈7-kb *G. lamblia* genomic DNA insert (pFDG417; lanes C and D) or carrying the 3-kb subclone (*D3*) used in these studies (pFDG417/D3; lane E) were induced with 10 mM isopropyl thio-D-galactoside (IPTG) (lanes B, D, and E) or were uninduced (lanes A and C). Extracts were electrophoresed in reducing SDS/8.5% PAGE (17), transferred to nitrocellulose (18), and treated with the rabbit antiserum raised against the Triton X-114 detergent-phase (14) extract of *G. lamblia* membrane.

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-205                                                    GAATTCTTACGCTATGTACGGCTTATATTGACAGGATTGCTACAGGCTATGAATACTATGCTAGAGTATAAACATGTA
-127  TCCACGGCGATCTGGGGTCTTCTCGGAGACTAGTGGCCAGTTACCATGGACACGCAAGAAGCTGTCTGTGGTAGCCTGGCCCCGGGCTTTGCGTTGGAAGCGCCACCCAGCAGGTCGGCGGCCTA
1     ATGTTCGGCAGATTTTTGCTCGCGATCGTCATCCTTCAGCTGGCACGGACAGCCTGCACCCAAGAAGCTGACGATGGAAAGTGTAAAACGTGTGGCGTCACCATTGGTCAAGACACTTGGTGCTCT
1      M  P  G  R  F  L  L  A  I  V  I  L  Q  L  A  R  T  A  C  T  Q  E  A  D  D  G  K  C  K  T  C  G  V  T  I  G  Q  D  T  W  C  S
127   GAGTGCAACGGAGCAAACTACGCCCCCGTGAACGGCCAGTGTGTAGACGTCAACGCTGAGGGGCCAAGCAAAACGCTTTGTCCGCAACATAGCGCAGGGAAGTGCACGCAGTGCGGAGGCAACTCA
43     E  C  N  G  A  N  Y  A  P  V  N  G  Q  C  V  D  V  N  A  E  G  P  S  K  T  L  C  P  Q  H  S  A  G  K  C  T  Q  C  G  G  N  S
253   TTCATGTACAAGGACGGCTGTTATTCCAGCGGAGAAGGCCTTCCTGGACACAGCCTGTGCTTAAGTTCCGACGGAGATGGCGTATGCACCGAGGCGGCCCCGGGGTACTTTGCTCCGGTGGGAGCG
85     F  M  Y  K  D  G  C  Y  S  S  G  E  G  L  P  G  H  S  L  C  L  S  S  D  G  D  G  V  C  T  E  A  A  P  G  Y  F  A  P  V  G  A
379   GCGAACACTGAACAGTCTGTGATCGCATGTGGCGATACAACTGGAGTAACAATAGCAGCTGGCGGAAACACATACAAGGGCATTGCTGACTGCGCAGAATGCAGCGCCCCTGACGCAACAGCCGGC
127    A  N  T  E  Q  S  V  I  A  C  G  D  T  T  G  V  T  I  A  A  G  G  N  T  Y  K  G  I  A  D  C  A  E  C  S  A  P  D  A  T  A  G
505   GCTGAGGCCGGCAAGGTTGCAACGTGTACCAAGTGTGGAGTCAGTAAGTATCTCAAGGATAACGTGTGCGTAGATAAAGCCCAATGTAATTCTGGTAGCACTAATAAGTTCGTTGCAGTTGATGAT
169    A  E  A  G  K  V  A  T  C  T  K  C  G  V  S  K  Y  L  K  D  N  V  C  V  D  K  A  Q  C  N  S  G  S  T  N  K  F  V  A  V  D  D
631   TCTGAGAATGGCAACAAGTGTGTTTCTTGCAGCGATAACCTCAATGGTGGCGTTGCCAATTGCGACACCTGTAGCTACGATGAGCAATCTAAGAAGATCAAGTGTACAAAATGCACCGATAACAAC
211    S  E  N  G  N  K  C  V  S  C  S  D  N  L  N  G  G  V  A  N  C  D  T  C  S  Y  D  E  Q  S  K  K  I  K  C  T  K  C  T  D  N  N
757   TACCTGAAAACCACAAGCGAAGGCACGTCGTGCGTACAAAAAGACCAATGCAAAGACGGCTTCTTCCCCAAGGATGACAGCAGTGCAGGAAATAAATGCCTCCCTTGTAATGACAGCACCGACGGA
253    Y  L  K  T  T  S  E  G  T  S  C  V  Q  K  D  Q  C  K  D  G  F  F  P  K  D  D  S  S  A  G  N  K  C  L  P  C  N  D  S  T  D  G
883   ATTGCCAATTGCGCCACGTGTGCTCTGGTTAGTGGCCGATCAGGGGCTGCCCTCGTTACATGCTCCGCCTGCACGGATGGATACAAGCCTAGTGCCGACAAAACTACGTGCGAGGCGGTAAGCAAC
295    I  A  N  C  A  T  C  A  L  V  S  G  R  S  G  A  A  L  V  T  C  S  A  C  T  D  G  Y  K  P  S  A  D  K  T  T  C  E  A  V  S  N
1009  TGCAAGACCCCCGGATGCAAGGCGTGCAGCAACGAAGGAAAGGAGAACGAGGTCTGCACAGACTGTGATGGTAGCACATACCTCACGCCGACAAGCCAGTGCATAGACAGCTGCGCTAAGATTGGA
337    C  K  T  P  G  C  K  A  C  S  N  E  G  K  E  N  E  V  C  T  D  C  D  G  S  T  Y  L  T  P  T  S  Q  C  I  D  S  C  A  K  I  G
1135  AACTACTATGGAGCCACCGAAGGAGCAAAGAAACTCTGTAAAGAGTGCACTGCGGCTAACTGCAAGACTTGCGATGATCAGGGGCAGTGCCAAGCATGCAACGACGGGTTCTATAAAAACGGCGAC
379    N  Y  Y  G  A  T  E  G  A  K  K  L  C  K  E  C  T  A  A  N  C  K  T  C  D  D  Q  G  Q  C  Q  A  C  N  D  G  F  Y  K  N  G  D
1261  GCGTGCTCTCCGTGCCACGAAAGCTGCAAGACATGCAGCGCAGGCACTGCCAGCGACTGCACCGAGTGTCCCACCGGAAAAGCACTCAGGTACGGGGACGACGGTACTAAGGGCACGTGCGGAGAA
421    A  C  S  P  C  H  E  S  C  K  T  C  S  A  G  T  A  S  D  C  T  E  C  P  T  G  K  A  L  R  Y  G  D  D  G  T  K  G  T  C  G  E
1387  GGCTGCACAACGGGCACAGGAGCAGGAGCATGCAAGACGTGTGGGCTCACTATCGATGGCGCTAGCTACTGCTCTGAGTGCGCCACAACGACAGAATATCCTCAAAATGGCGTCTGTGCACCAAAG
463    G  C  T  T  G  T  G  A  G  A  C  K  T  C  G  L  T  I  D  G  A  S  Y  C  S  E  C  A  T  T  T  E  Y  P  Q  N  G  V  C  A  P  K
1513  GCTAGCCGCGCCACACCTACGTGCAACGACTCGCCTATTCAGAATGGTGTTTGTGGAACGTGTGCCGATAACTACTTTAAGATGAACGGAGGGTGCTATGAAACAGTCAAGTATCCCGGTAAGACG
505    A  S  R  A  T  P  T  C  N  D  S  P  I  Q  N  G  V  C  G  T  C  A  D  N  Y  F  K  M  N  G  G  C  Y  E  T  V  K  Y  P  G  K  T
1639  GTTTGCATTAGTGCACCAAATGGTGGTACGTGTCAAAAAGCTGCAGATGGTTACAAGTTGGATTCAGGTACCCTTACAGTTTGTTCTGAAGGGTGTAAGGAATGTGCTAGCAGTACCGACTGTACT
547    V  C  I  S  A  P  N  G  G  T  C  Q  K  A  A  D  G  Y  K  L  D  S  G  T  L  T  V  C  S  E  G  C  K  E  C  A  S  S  T  D  C  T
1765  ACGTGTCTGGACGGATATGTAAAGAGTGCAAGTGCGTGCACAAAGTGTGACGCTAGCTGCGAAACATGTAATGGAGCAGCTACAACATGTAAGGCGTGTGCTACGGGATACTACAAGACCGCATCA
589    T  C  L  D  G  Y  V  K  S  A  S  A  C  T  K  C  D  A  S  C  E  T  C  N  G  A  A  T  T  C  K  A  C  A  T  G  Y  Y  K  T  A  S
1891  GGAGAAGGTGCGTGCACGTCTTGTGAAAGTGATAGCAACGGAGTCACTGGTATTAAGGGCTGCCTAAACTGCGCCCCTCCGCCCAACAATAAAGGTTCCGTCCTCTGCTACCTCATAAAGGATAGC
631    G  E  G  A  C  T  S  C  E  S  D  S  N  G  V  T  G  I  K  G  C  L  N  C  A  P  P  P  N  N  K  G  S  V  L  C  Y  L  I  K  D  S
2017  GGTAGCACCAACAAGAGCGGGCTCTCCACTGGTGCCATAGCGGGTATCTCCGTCGCTGTCATCGTTGTTGTCGGCGGCCTCATCGGCTTCCTCTGCTGGTGGTTCCTCTGCAGGGGGAAGGCGTAG
673    G  S  T  N  K  S  G  L  S  T  G  A  I  A  G  I  S  V  A  V  I  V  V  V  G  G  L  I  G  F  L  C  W  W  F  L  C  R  G  K  A  *
2143  ATGTACTTAGATAGTAAACCGTCATCGATGGGTCTGCTCGGTGTCTGTTCCTGC
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FIG. 2. DNA and translated amino acid sequence of gene *TSA 417*. Putative TATA box (24), Shine–Dalgarno ribosomal binding sites (25) preceding the first two methionine residues, and the AGTRAA (26) sequence after *Giardia* structural genes are underlined. The three possible N-glycosylation sites are at positions 289, 513, and 676, and the 29 repeats of the Cys-Xaa-Xaa-Cys motif are underlined. The amino-terminal signal peptide (27) and carboxyl-terminal membrane-spanning regions (28) are boxed. The DNA sequence was translated using the DNASIS program (LKB).

bases from the beginning of the insert (Fig. 2) and is preceded by a prokaryotic Shine–Dalgarno ribosomal binding sequence (beginning at −17) (25).

Possible control elements in the 5′ untranslated region of the *TSA 417* gene include a presumptive TATA box (24) beginning at position −134 (Fig. 2). The 3′ untranslated region contains the canonical AATAAA polyadenylylation sequence (29) at 2626 (not shown). Moreover, the sequence AGTRAA, in which R is an adenine or guanine residue and which is present six to nine nucleotides downstream of the stop codon of all *Giardia* protein-encoding genes reported to date (26), is also present 12 nucleotides beyond the stop signal of the *TSA 417* gene (Fig. 2).

The peptide molecular mass predicted by the translated amino acid sequence is 72.5 kDa, and its composition is strikingly rich in cysteine. Of 713 residues, 84 or 11.8% are cysteine, and the motif Cys-Xaa-Xaa-Cys is repeated 29 times dispersed throughout the protein (Fig. 2). Regions of proteins with potential homology (44) identified by searches of the GenBank (Release 60) and Protein Identification Resource (Release 20) data bases (30) consisted largely of this motif.

The deduced amino acid sequence of TSA 417 (Fig. 2) reveals a classical amino-terminal signal peptide (27). TSA 417 is hydrophilic (mean hydropathy index = −0.3), but it has a single strongly hydrophobic region (28) of 30 amino acids near the carboxyl terminus.

Characterization of the TSA 417 Antigens. The biologic importance of TSA 417 is strongly supported by the observation that antiserum against the recombinant proteins reacts with live trophozoites as shown by immunofluorescence and agglutinates them (Fig. 3). Neither fluorescence nor agglutination was observed with either control (anti-*E. coli*) or preimmune serum (not shown). Moreover, immunoelectron microscopy (Fig. 4) confirms that the rabbit anti-TSA 417 serum reacts with the entire trophozoite surface, including the outer face of the plasma membrane surrounding the flagella and the adhesive disc.

In standard microscopic assays, heat-inactivated anti-TSA 417 antiserum (20%) strongly agglutinated live trophozoites and (at 40%) inhibited their attachment by >85%, compared with control sera, but did not lyse them. Moreover, anti-TSA 417 totally inhibited trophozoite growth. When 3.2×10^5

FIG. 3. Reaction of live trophozoites with anti-recombinant antiserum. Antibodies against recombinant TSA 417 were allowed to react with live trophozoites. After a washing, bound antibody was detected by reaction with fluorescein isothiocyanate-conjugated goat-antibody to rabbit immunoglobulins.

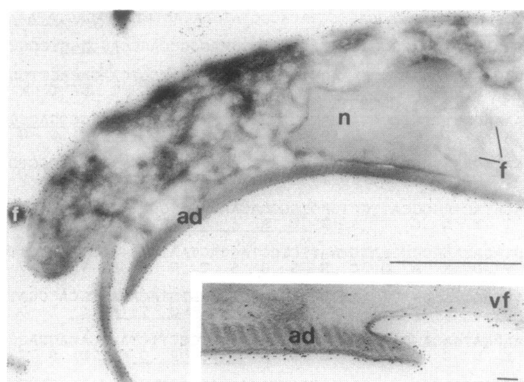

FIG. 4. Antibodies against recombinant TSA 417 react with the entire trophozoite outer surface by frozen section immunoelectron microscopy (22). The obliquely sectioned flagella next to the nucleus are within the cell body and are not bounded by membrane, in contrast to the single anterior flagellum. Only the latter is labeled with anti-TSA 417. The flagellar ultrastructure is less distinct in frozen sections. (Bar = 1 μM.) (*Inset*) Immunogold label on the outer face of the plasma membrane over the ventral adhesive disc. (Bar = 0.1 μm.) ad, Adhesive disc; f, flagellum; n, nucleus; vf, ventral flange.

trophozoites per ml were incubated at 37°C with 20% heat-inactivated antiserum, only 1.3×10^5 cells per ml were counted after 18 hr, compared with 6.9×10^5 per ml for the anti-*E. coli* serum control.

The antiserum against recombinant TSA 417 immunoprecipitated the major 66-kDa antigen and an 85-kDa protein, which is either less prevalent or less exposed, from extracts of surface-iodinated *G. lamblia* trophozoites (Fig. 5, lanes D and E). The 85-kDa band was more prominent in immunoprecipitates of metabolically labeled trophozoites (Fig. 5, lane B). As predicted by the amino acid sequence, the 66- and 85-kDa species represent major [^{35}S]cysteine-labeled bands in whole-cell extracts (Fig. 5, lane A) and were the only labeled proteins precipitated by anti-TSA 417 (Fig. 5, lane B) but were not precipitated by preimmune serum (lanes C and F) or by control anti-*E. coli* serum (not shown). Moreover, they are abundant parasite proteins, since they are visible in gels of unlabeled Triton X-114 detergent-phase extracts stained with Coomassie blue (not shown).

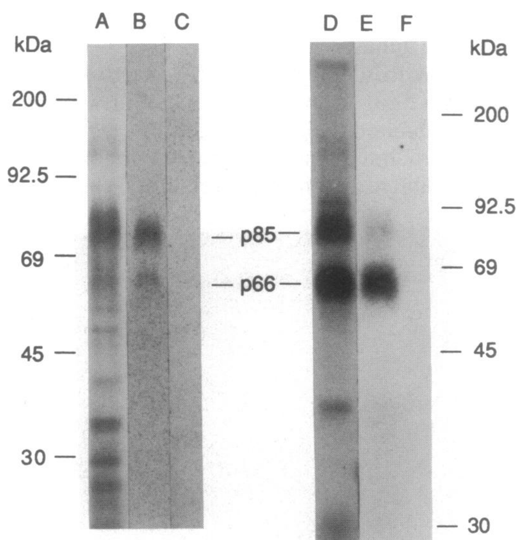

FIG. 5. Metabolic labeling with [^{35}S]cysteine or surface-labeling with ^{125}I. Extracts of trophozoites that had been metabolically labeled with [^{35}S]cysteine (lanes A, B, and C) or surface-labeled with ^{125}I (lanes D, E, and F) were separated on SDS/8.5% PAGE under reducing conditions. Lanes: A and D, total extract; B and E, trophozoite extract immunoprecipitated with antibody raised against recombinant TSA 417; C and F, trophozoite extract immunoprecipitated with preimmune serum.

DISCUSSION

It is likely that the great variations in duration and severity of disease in people infected with *Giardia* are due to interactions of trophozoite surface molecules with immune and nonimmune components of the host intestinal milieu. To better understand how surface molecules enable the trophozoite to withstand this hostile environment, we have used an antiserum against a Triton X-114 (14) trophozoite membrane extract to isolate the gene, called *TSA 417,* which encodes two major TSA species.

Antibodies against TSA 417 expressed in *E. coli* agglutinate live trophozoites, react with the outer surface of their plasma membrane, as shown by immunofluorescence and immunoelectron microscopy, and immunoprecipitate the major 66- and 85-kDa surface-labeled TSAs. Moreover, we have shown that the antibody to recombinant TSA 417 inhibits both trophozoite attachment and growth. Agglutinating antibody would be an important protective mechanism (31), since it would crosslink flagella to each other or to the disc and physically interfere with both motility and cytokinesis, even if the target antigen were not directly involved in these processes. We have observed that TSAs of 85 and 66 kDa are recognized by antibodies from serum and milk of some patients (not shown). In the intestinal tract, secretory antibodies (sIgA and sIgM) would be especially likely to crosslink/agglutinate because of their high valency (31). The relationship between the 85-kDa molecule, whose gene we have cloned, and major surface-iodinated TSAs of $\approx$88 (32) or 82 (33–35) kDa, which are recognized by patient sera, remains to be determined.

The deduced TSA 417 sequence shows a typical amino-terminal signal peptide that probably targets the protein for insertion into and translocation across the plasma membrane (27). The hydropathy profile of TSA 417 shows a slightly hydrophilic protein with a single strongly hydrophobic membrane-spanning region (28) followed by a short charged region (Cys-Arg-Gly-Lys-Ala) at the carboxyl terminus. These observations, together with the antibody, surface labeling and Triton X-114 extraction studies, suggest that, although most of the protein is on the outer surface of the trophozoite, it is anchored in the plasma membrane by this hydrophobic region, followed by a short, charged cytoplasmic "tail." The carboxyl-terminal peptide does not appear to be replaced by a glycophosphatidylinositol anchor (36), since the 66-kDa and 85-kDa TSAs are not among the trophozoite proteins labeled with tritiated fatty acids (S. Das, private communication).

Giardia has been reported to be one of the earliest organisms to diverge from the eukaryotic line of descent (26, 37, 38). Therefore, studies such as those reported here may yield new insights into the evolution of certain eukaryotic cellular structures or functions. For example, the *TSA 417* gene has a bacterial ribosome binding site (25), and the complement to the Shine–Dalgarno sequence in *Giardia* rRNA has been reported (38, 39). Moreover, this gene has none of the consensus nucleotides surrounding the ATG codon that have been reported by Kozak (40) to be important for the initiation of translation by eukaryotic ribosomes. The presence of a bacterial ribosomal binding site may explain the efficient expression of the *G. lamblia TSA 417* gene in *E. coli* without requiring protein fusion. Moreover, the *TSA 417* gene expresses prominent 85- and 66-kDa *Giardia* surface membrane antigens in both the parasite and in recombinant *E. coli*. This suggests that any conformational, processing, and/or modification signals within the protein may be recognized in both

organisms. The recognition of information in *TSA 417* DNA and protein sequences by *E. coli* is also consistent with the early position of *Giardia* in the evolution of eukaryotes (38, 39).

The similarity in expression of the 417 gene in *E. coli* and in *G. lamblia* supports the idea that both the 66- and 85-kDa TSAs are products of the *TSA 417* gene. Since recombinant eukaryotic proteins do not appear to be glycosylated or fatty-acylated in bacteria, the 85-kDa species probably corresponds to the primary 72.5-kDa product of the *TSA 417* gene. Its migration in SDS/PAGE may be anomalously slow because of its highly cysteine-rich composition (19, 44). The TSA 66-kDa might be a product of proteolytic processing of the 85-kDa TSA or another stable conformation of the same polypeptide.

The importance of the strikingly high cysteine content (11.8%) of TSA 417 is supported by previous studies that demonstrated the crucial role of this amino acid in the biology of *Giardia*. This parasite is unusual in its requirement for cysteine at millimolar concentrations for survival, growth, and attachment *in vitro* (41). Furthermore, we have demonstrated reduced −SH groups exposed on the outer surface of trophozoites by flow cytometry with a thiol-specific fluorescent hapten (42). These −SH groups appeared to be crucial to trophozoite survival, since treatment with nonpenetrating thiol blockers was lethal (42). Surface thiols may protect trophozoites from oxygen or from free radicals (43) and may be involved in interaction with host intestinal epithelial cells. A previously described cloned 33-kDa peptide fragment of a 170-kDa *Giardia* surface protein also contains 12% cysteine (44) with the Cys-Xaa-Xaa-Cys motif occurring 15 times, indicating possible common ancestry or function with TSA 417. This 170-kDa variable species (19) has not been detected in our parasites (Fig. 5).

Cysteine-rich domains on outer-membrane proteins of *Chlamydia* have been observed to be highly conserved and to promote structural stability by forming intra- and intermolecular disulfide bridges (45). If TSA 417 molecules on the trophozoite surface are also highly crosslinked by disulfide bonding, this may help to explain (46) how *G. lamblia* thrives in the extremely degradative, protease- and detergent-rich environment of the human small intestine.

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