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Conformationally Appropriate Expression of the Toxoplasma Antigen SAG1 (p30) in CHO Cells

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The *Toxoplasma gondii* major surface antigen, called SAG1 or p30, is a highly immunogenic protein which has generated great interest as a diagnostic reagent, as a potential subunit vaccine, and for its role in invasion. Unfortunately, bacterial recombinant protein is grossly misfolded so that, for example, it is not effectively recognized by antibodies to native SAG1. To overcome this, we have turned to expression in CHO cells, using cotransfection of the *SAG1* gene and the mouse dihydrofolate reductase (DHFR) gene into CHO cells that are DHFR$^-$. SAG1 expression was amplified by methotrexate coselection of CHO cells in combination with fluorescence-activated cell sorting for SAG1 expression. The resulting population expressed recombinant SAG1 that is recognized by antiserum specific for natural, nonreduced SAG1, indicating that, unlike in bacteria, expression in CHO cells results in proper folding. Processing was at least partially correct in that, like natural SAG1, recombinant SAG1 was attached to the plasma membrane via a glycolipid anchor, although tunicamycin treatment was necessary to prevent N-glycosylation (SAG1 is not glycosylated in the parasite but does have a consensus N-linked site). Finally, purified recombinant SAG1 was recognized by human sera known to be reactive to toxoplasma proteins, indicating that this material has potential as a diagnostic reagent and possibly as a component of a subunit vaccine.

Toxoplasma gondii, an obligate intracellular protozoan parasite, has long been associated with severe congenital defects and life-threatening infection in immunocompromised hosts. In the past decade, it has received increased attention as a common opportunistic pathogen which affects up to 25% of AIDS patients (5, 16). Many investigators have focused on efforts to improve diagnostic testing for toxoplasmosis and to develop a subunit vaccine which could prevent or attentuate disease. Much of this work has centered upon SAG1, or p30, the major surface antigen of toxoplasma tachyzoites.

Recent studies indicate that SAG1 may play an important role in invasion of host cells (8, 17). Unlike surface antigens of other protozoa, such as plasmodia and trypanosomes, SAG1 is a nonvariant antigen which is well conserved immunologically (30) and at the sequence level (2, 26).

SAG1 elicits a robust immune response in infected animals, including humans, and immunization with purified SAG1 protects mice from a lethal parasite challenge (2, 14). This immunity appears to be primarily mediated by CD8$^+$ cells specific for SAG1 (13, 14). Humoral immunity also plays a significant role in host defenses against the parasite. Monoclonal antibodies to SAG1 can be partially protective in the mouse model (12), and polyclonal and some monoclonal antibodies to SAG1 can also prevent infection of host cells in vitro (8, 17).

SAG1 is a highly conformational antigen, whose recognition by antibody is dependent upon correct disulfide bonding. As a result, antiserum to native SAG1 generally does not efficiently recognize denatured or reduced SAG1 (3). Given the potential contribution of humoral immunity in host defenses against toxoplasma and SAG1's proven efficacy as a protective antigen, an optimal subunit vaccine against toxoplasmosis would incorporate recombinant SAG1, ideally, in its natural conformation.

Recombinant SAG1 is also likely to be an invaluable diagnostic reagent. Currently, diagnostic testing is based almost exclusively on serological testing using parasite antigens which can only be obtained in sufficient amounts by harvesting parasites from vast numbers of infected mice. In humans, serum reactivity to SAG1 is one of the earliest and most consistent signs of toxoplasma infection (21–24), and several investigators have shown that the presence of immunoglobulin A (IgA) to SAG1 is highly indicative of acute infection (6, 11).

Because of SAG1's potential as a diagnostic reagent and as a component of a subunit vaccine, we have expressed and characterized recombinant SAG1 with the aim of producing a recombinant protein which is folded and processed similarly to native SAG1. Eukaryotic expression is widely used for expression of mammalian or viral proteins but has not been used extensively for antigens from lower eukaryotic pathogens such as toxoplasma. Most of the extensively studied antigens of protozoal pathogens are conformational surface antigens which, like SAG1, undergo complex processing, such as disulfide bonding, glycosylation, or glycolipid anchor addition, as they transit the secretory pathway to the cell surface. This study shows that expression in CHO cells of a parasite antigen such as SAG1 results in efficient production of large amounts of recombinant protein which is correctly folded and targeted. We anticipate that this approach, particularly in conjunction with fluorescence-activated cell sorting (FACS) selection and amplification, should prove helpful to the study of the surface antigens of other pathogenic protozoa.

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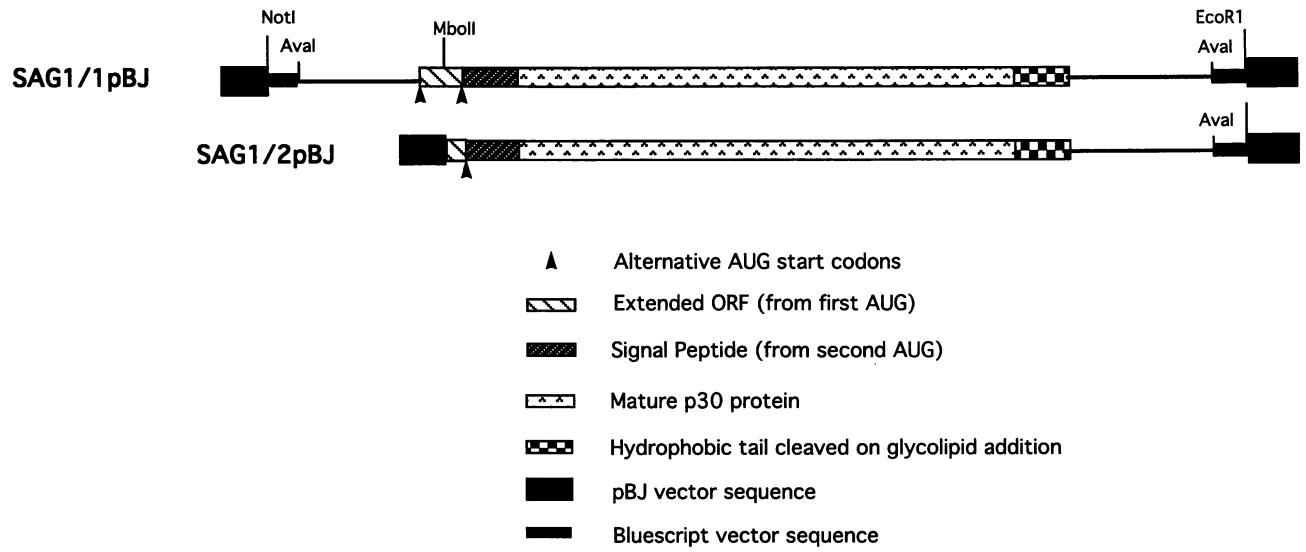

FIG. 1. SAG1 eukaryotic expression constructs. SAG1/1pBJ and SAG1/2pBJ were constructed from pBJ_5 and an *Ava*I SAG1 genomic fragment as described in Materials and Methods. (The figure is not to scale; see Materials and Methods for lengths of fragments indicated.) Sites of processing of the SAG1 protein are indicated, as are the *Not*I, *Eco*RI, and *Mbo*II sites used for cloning. The fragments between *Not*I and *Ava*I and between *Eco*RI and *Ava*I are derived from the BluescriptII vector from which the *Ava*I fragment was obtained. ORF, open reading frame.

MATERIALS AND METHODS

SAG1 constructs. The 1.6-kb genomic *Ava*I fragment of the *SAG1* gene, which contains the entire coding region (3), was originally cloned into the *Sma*I site of BluescriptII KS+. The genomic fragment contains approximately 360 bp of 5′ untranslated sequence, 310 bp of 3′ untranslated sequence, and 960 bp of coding sequence (which encodes a signal peptide of approximately 30 amino acids and a 25- to 30-amino-acid hydrophobic C terminus, presumably cleaved upon glycolipid addition). This fragment was cut from the plasmid by using the *Not*I and *Eco*RI polylinker sites flanking the insert and cloned into the polylinker of *Not*I-*Eco*RI-digested pBJ_5 (gift of Dan Denny, Stanford University), a derivative of the SRα series of vectors (28). This plasmid is referred to as SAG1/1pBJ (Fig. 1). A second construct was made by deleting a 360-bp *Not*I-*Mbo*II fragment, filling in with Klenow fragment, and religating. In this construct, SAG1/2pBJ (Fig. 1), the first ATG following the transcription start site in the genomic clone was removed (3).

mDHFR/SK is Bluescript SK− with the mouse dihydrofolate reductase (DHFR) minigene (7) cloned into the *Eco*RI and *Pst*I sites. This construct was used in control transfections of CHO cells.

Tissue culture and selection of SAG1 recombinants. RH strain toxoplasma tachyzoites were maintained in human foreskin fibroblasts grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL) supplemented with 10% Nuserum (Collaborative Research), 2 mM glutamine, and 20 μg of gentamicin per ml. Parasites were harvested from lysed host cell monolayers by filtration through 3.0-μm Nucleopore filters as described previously (25).

CHO K1 DUX-B11 cells, lacking the DHFR gene, were maintained in Ham's F12 complete medium supplemented with 2 mM glutamine, 20 μg of gentamicin per ml, and 10% fetal calf serum (FCS). Approximately 4×10^7 CHO cells were washed and resuspended in 900 ml of HeBS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 137 mM NaCl, 5 mM KCl, 0.07 mM Na_2HPO_4, 6 mM glucose [pH 7.0]). Linearized DNA (16 μg of SAG1/2pBJ plus 4 μg of mDHFR/SK or 4 μg of mDHFR/SK alone) resuspended in 100 ml of HeBS was mixed with cells in a 4-mm gap cuvette. After incubation for 10 min on ice, the cells were electroporated in a Bio-Rad Gene Pulser set at 0.23 kV, with the capacitance extender at 960 mF, which elicited a time constant of 18 ms. After electroporation, the cells were incubated on ice for 10 min and then transferred to a T175 flask containing complete F12 medium. After 3 days, the medium was changed to selective F12 medium lacking hypoxanthine, thymidine, and glycine and supplemented with 10% dialyzed FCS, 2 mM glutamine, and 20 μg of gentamicin per ml. The medium was changed every 2 days. Cells transformed with a mixture of SAG1/2pBJ and mDHFR/SK were designated CHO/SAG1, whereas cells transformed with mDHFR/SK alone were designated CHO/DHFR.

Cells able to grow in selective medium were adapted to DMEM supplemented with 10% dialyzed FCS, 2 mM glutamine, and 20 μg of gentamicin per ml (complete medium). CHO/SAG1 cells were exposed to fourfold increases in methotrexate beginning at a concentration of 0.02 μM, alternating with rounds of FACS for determination of SAG1 surface expression (see below). Methotrexate selection was terminated when a population of cells able to grow in 100 μM methotrexate had been obtained. CHO cells to be used for experiments or affinity purification were incubated in medium supplemented with 10 mM sodium butyrate for approximately 16 h before harvesting (15).

FACS analysis. Live CHO cells were removed from tissue culture flasks by incubation with phosphate-buffered saline (PBS)–1 mM EDTA for 20 min at 37°C. Cells were spun at $400 \times g$ for 5 min, resuspended in DMEM–1% FCS, and incubated on ice for 20 min. The cells were then resuspended in DMEM–1% FCS containing DG52 ascites diluted 1:500 for 30 min on ice. DG52 is a mouse monoclonal antibody

which recognizes nonreduced SAG1 only (2, 3). After being washed twice in PBS–1 mM EDTA–1% FCS, the cells were incubated in DMEM–1% FCS containing fluorescein isothiocyanate-conjugated goat anti-mouse Ig antiserum (Cappel) diluted 1:500 and 1 μg of propidium iodide per ml for 30 min on ice. Cells were washed twice and sorted on a Facstar fluorescence-activated cell sorter (Becton Dickinson). Secondary antibodies used for immunofluorescence or Western blotting (immunoblotting) were checked before use in experiments to verify lack of reactivity to toxoplasma proteins.

Western blotting. Cells were lysed in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1% glycerol, 1.5% SDS, 125 mM Tris-HCl [pH 6.8], 0.05% bromphenol blue) and resolved on a 12% polyacrylamide–SDS gel under nonreducing conditions. The gel was transferred to a nitrocellulose membrane, blocked with PBS–1% nonfat dry milk–0.05% Tween 20 and incubated with antibody diluted in blocking solution for 1 h at room temperature. After being washed three times with PBS–0.05% Tween 20, the blot was incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Cappel) diluted 1:1,000 in blocking solution. The membrane was washed three times with PBS–0.05% Tween 20 and processed with the ECL chemiluminescence detection kit (Amersham) as per the manufacturer's instructions. Antibodies specific for native or nonreduced SAG1 included mouse monoclonal antibodies DG52 (1:1,000) and 1E11 (1:250; a gift from Jack Remington, Palo Alto Medical Foundation) and rabbit anti-SAG1 serum (1:500; a gift from Lloyd Kasper, Dartmouth University). Individual human patient sera previously tested by the Sabin-Feldman dye test (Palo Alto Medical Foundation; negative and positive sera ranged in titer from 1:32 to 1:1,024) were used at a dilution of 1:50; processing of blots was identical except that 5% milk was substituted for 1% milk in the blocking solution. The isotype reactivity of the human samples was not known; the samples were presumably from chronically infected individuals, but adequate clinical information was not available.

Immunoaffinity purification. DG52 (10 mg) was purified from mouse ascites fluid on a protein A-Sepharose column (Pharmacia). This was coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. After coupling, the column was equilibrated with PBS–0.02% azide and stored at 4°C. Before use, the column was equilibrated with PBS–0.5% Nonidet P-40.

All steps of the immunoaffinity purification of SAG1 were performed at 4°C. Purified parasites or CHO cells harvested from roller bottles with PBS–1 mM EDTA were centrifuged at 4°C for 15 min at 400 × *g*. The pellet was lysed on ice for 15 min in PBS–0.5% Nonidet P-40 containing protease cocktail (aprotinin, leupeptin, E-64, chymostatin, and pepstatin, all at a final concentration of 5 μg/ml). Parasites were resuspended at 5×10^8/ml, and CHO cells were resuspended at 10^8/ml. The suspension was centrifuged at 37,500 × *g* for 30 min at 4°C. The resulting supernatant was loaded on the DG52 column and allowed to circulate on the column for 2 h. The column was washed with 10 volumes of PBS–0.5% Nonidet P-40 and then with 10 volumes of 50 mM Tris-HCl (pH 8.0)–1 M NaCl–1% *N*-octyl glucoside. SAG1 was eluted from the column with 50 mM sodium phosphate (pH 12)–1% *N*-octyl glucoside made just prior to use. Fractions (0.5 ml) were collected and immediately neutralized with 50 μl of 0.5 M HEPES (pH 6.5). The column was washed with 10 volumes of 0.5 M HEPES (pH 6.5)–0.5% Nonidet P-40, 10 volumes of PBS–0.5% Nonidet P-40, and 10 volumes of PBS–0.02% azide and stored. Fractions were tested by Western blot for SAG1. Immunoaffinity-purified SAG1 was sometimes contaminated with small amounts of antibody from the column.

***N*-Glycanase treatment.** Affinity-purified recombinant SAG1 was denatured by boiling in 50 μl of 0.5% SDS at 100°C for 10 min. Then 5 μl of 0.5 M sodium phosphate buffer (pH 7.6) and 5 μl of 10% Nonidet P-40 were added, followed by 1 μl of peptide *N*-glycanase F (1,000 U/ml; New England Biolabs). The reaction mix was incubated at 37°C for 1 h. *N*-Glycanase-treated samples, mock-treated samples, and affinity-purified SAG1 from tachyzoites were electrophoresed on a 12% polyacrylamide–SDS gel. After transfer to nitrocellulose, a Western blot was performed with DG52 (1:1,000).

Metabolic labeling and immunoprecipitation. For [^{3}H]leucine labeling, T25 flasks of 50 to 75% confluent CHO/SAG1 or CHO/DHFR cells were washed with serum-free medium lacking leucine. Cells were labeled overnight in leucine-free medium supplemented with 20 μCi of L-[4,5-^{3}H]leucine (Amersham) per ml, 10 mM sodium butyrate, and 10% complete medium. For [^{3}H]ethanolamine labeling, cells were incubated in complete medium with 100 to 150 μCi of [1-^{3}H]ethanolamine (Amersham) per ml and 10 mM sodium butyrate.

Cells were lysed with 1 ml of RIPA (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1% Nonidet P-40 0.5% deoxycholate, 0.1% SDS) supplemented with protease cocktail (see above) on ice for 30 min. The lysate was spun in a microcentrifuge at 14,000 × *g* for 10 min. DG52 ascites (10 μl) was added to the supernatant, and the suspension was incubated at 4°C. Protein A-Sepharose beads (20 μl, wet volume) were added after 1 h, and the suspension was incubated for another hour. The beads were washed five times with RIPA, and samples were eluted with 1× SDS-PAGE sample buffer and loaded onto a 12% polyacrylamide–SDS gel. After electrophoresis, the gel was fixed, treated with Entensify (Dupont-NEN), dried, and exposed to XAR (Eastman Kodak) film.

PI-PLC treatment. Phosphatidylinositol-specific phospholipase C (PI-PLC) purified from *Bacillus thuringiensis* (2,000 U/ml) was a gift from Suzanne Eriksson, Stanford University. CHO cells were labeled with leucine as above except that the medium was supplemented with 0.2 U of PI-PLC per ml. The next day, the medium and cell monolayer (washed with PBS) were immunoprecipitated as above.

RESULTS

Expression of SAG1. Our goal was to obtain recombinant SAG1 that resembled the natural protein as closely as possible and that was efficiently recognized by immune sera. We first attempted bacterial expression of SAG1 in a variety of bacterial expression systems, including the nonfusion systems pOTS and pET (based on T7 polymerase) and the fusion systems pATH (TrpE), pGEX (glutathione-*S*-transferase), and pRIT (protein A). SAG1 was poorly expressed as a nonfusion protein, and although expression was more efficient in the fusion systems, none produced protein which was efficiently recognized by antiserum generated against native SAG1 (data not shown). We assume that the highly reducing environment of the *Escherichia coli* cytosol prevented the disulfide bonding necessary for recognition of recombinant SAG1 by our antiserum. (The importance of disulfide bonding in SAG1 can also be inferred from the significantly slower mobility of reduced than of nonreduced SAG1 and the fact that antibodies to native SAG1 show more than a 100-fold-lower reactivity against reduced SAG1

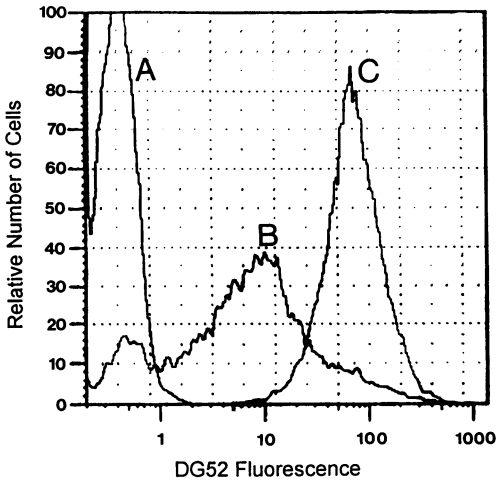

FIG. 2. FACS analysis of recombinant CHO cells. Live CHO cells were labeled with the anti-SAG1 monoclonal antibody DG52 as described in the text. (A) CHO/DHFR cells; (B) CHO/SAG1 cells growing in selective medium without methotrexate; (C) CHO/SAG1 cells after four rounds of FACS selection and methotrexate amplification.

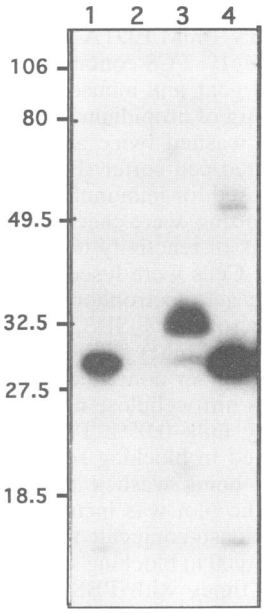

FIG. 3. Western blot of CHO/SAG1 cells. Samples were run on a 12% polyacrylamide–SDS gel, transferred to nitrocellulose, and probed with DG52 diluted 1:1,000. Lane 1, affinity-purified SAG1 from toxoplasma tachyzoites; lane 2, CHO/DHFR lysate; lane 3, CHO/SAG1 lysate; lane 4, toxoplasma tachyzoite lysate. The positions of prestained size standards (Bio-Rad) are shown (in kilodaltons).

and vice versa [11].) We therefore decided to pursue eukaryotic expression of SAG1, reasoning that SAG1 was more likely to be accurately processed and folded if it passed through the secretory pathway of a eukaryotic cell.

We used cotransfection of DHFR with our SAG1 gene to facilitate high levels of SAG1 expression. In this system, exposure of transfected cells to gradually increasing concentrations of methotrexate results in the amplification of DHFR (7) and any other genes that have been cotransfected with DHFR. By using the SRα series of vectors, which contain a modified simian virus 40 promoter and a simian virus 40 origin of replication (28), we could test our constructs in transient-transfection assays in Cos cells before undertaking the lengthy process of methotrexate amplification in CHO cells.

SAG1/1pBJ and SAG1/2pBJ were tested in transient-transfection assays in Cos cells. Only transfection with SAG1/2pBJ (which had the first ATG deleted) resulted in high levels of expression of SAG1 in Cos cells detectable by immunofluorescence and Western blot (data not shown). This argued that although the first ATG is in frame, the second is the correct start site in vivo, as has recently been confirmed by transfection of the relevant constructs into toxoplasma (27). Transfections of CHO cells were therefore performed with SAG1/2pBJ.

CHO/SAG1 cells, obtained after cotransfection of CHO cells with SAG1/2pBJ and mDHFR/SK and selection of DHFR-positive cells, were labeled with monoclonal antibody DG52 and analyzed by FACS. Although the levels of SAG1 expression were low, comparison with similarly labeled CHO/DHFR cells clearly indicated that the transformants were expressing SAG1 and that, as these were live cells, at least some of this expression was on their surface (Fig. 2, lines A and B). To amplify SAG1 expression, serial rounds of FACS and methotrexate selection were performed. This protocol led to an approximately 10-fold increase in SAG1 expression after four rounds of amplification (Fig. 2, line C). We found that FACS alone was not sufficient for the selection of a population of cells stably expressing high levels of SAG1 and that methotrexate selection was also necessary. As has been found for other eukaryotic cells expressing recombinant proteins, we found that 10 mM sodium butyrate enhanced SAG1 expression when added to cultures the night before harvesting (15).

Characterization of recombinant SAG1. Most of the recombinant SAG1 migrated as a band apparently 4 to 5 kDa larger than the natural protein on SDS-polyacrylamide gels (Fig. 3). All polyclonal and monoclonal sera specific for native SAG1 recognized recombinant SAG1, suggesting that the protein was correctly folded (although reactivity to 1E11 was somewhat reduced compared with that of native SAG1; data not shown). As the SAG1 gene encodes a consensus N-linked glycosylation site (Asn-Xxx-Ser/Thr) which is not used by the parasite (19), the most likely explanation for the apparent size increase was that this site is being used in the CHO cells. To test this, purified recombinant SAG1 was treated with *N*-glycanase; this resulted in protein that comigrated with natural SAG1 from tachyzoites (Fig. 4A), as did tunicamycin treatment of CHO/SAG1 cultures (data not shown). We conclude that the difference in migration between recombinant and native SAG1 is indeed due to N-glycosylation at the consensus site in SAG1.

Native SAG1 is glycolipid anchored in toxoplasma (18, 29). We wished to verify that the recombinant SAG1 also contained a glycolipid anchor, as suggested by its expression on the surface of the recombinant CHO cells. (Proteins whose nucleotide sequences encode a hydrophobic carboxy terminus but are not appropriately processed to include glycolipid anchors are usually not trafficked to the surface of cells [4].) We confirmed the presence of a glycolipid anchor by two methods: treatment with PI-PLC, an enzyme specific for glycolipid linkages, and metabolic labeling. Treatment of [^{3}H]leucine-labeled CHO/SAG1 cells with PI-PLC led to

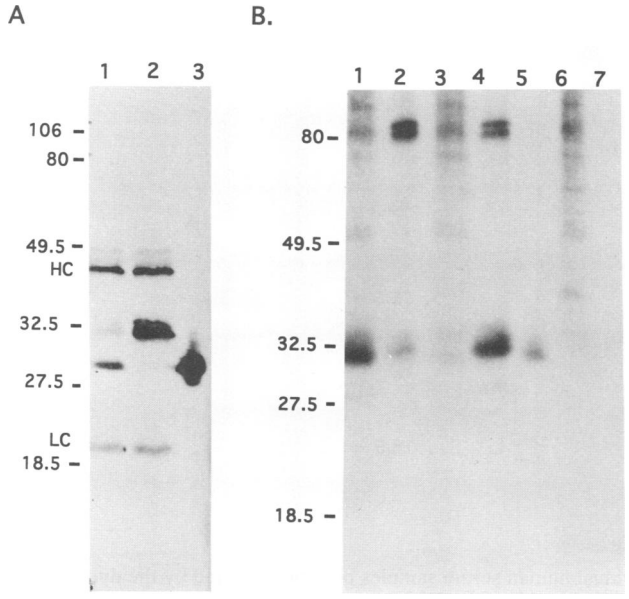

FIG. 4. Processing of recombinant SAG1. Samples were run on a 12% polyacrylamide–SDS gel, transferred to nitrocellulose, and probed with DG52 diluted 1:1,000. (A) *N*-glycanase treatment. Lane 1, peptide *N*-glycanase F-treated affinity-purified recombinant SAG1; lane 2, untreated affinity-purified recombinant SAG1; lane 3, affinity-purified SAG1 from toxoplasma tachyzoites. IgG heavy and light chains (eluted from the immunoaffinity column during purification of recombinant SAG1) are indicated by HC and LC, respectively. Heavy and light chains are not evident in lane 3 because the relative yield of SAG1 per milliliter of eluate from the column was higher. (B) PI-PLC cleavage and ethanolamine labeling of recombinant SAG1. All lanes are the immunoprecipitate obtained with the anti-SAG1 monoclonal antibody DG52. Lane 1, [^{3}H]leucine-labeled CHO/SAG1 cells; lane 2, medium from culture used for lane 1; lanes 3 and 4, as for lanes 1 and 2 except that CHO/SAG1 cells were treated with PI-PLC; lane 5, [^{3}H]ethanolamine-labeled CHO/SAG1 cell lysate; lanes 6 and 7, CHO/DHFR control cells labeled with [^{3}H]leucine and [^{3}H]ethanolamine, respectively. Sizes are shown in kilodaltons.

release of recombinant SAG1 into the culture medium, as verified by immunoprecipitation (Fig. 4B, lanes 1 to 4). While not all glycolipid anchors can be cleaved by PI-PLC, sensitivity to PI-PLC is considered diagnostic for proteins that are anchored to the plasma membrane via a glycophosphatidylinositol. In addition, recombinant SAG1, like native SAG1 (29), could be metabolically labeled with ethanolamine, an essential component of glycolipid anchors which is not incorporated into any of the other potential modifications seen on eukaryotic proteins (Fig. 4B, lane 5). Hence, we conclude that the recombinant material is glycophosphatidylinositol anchored.

Human immune serum reactivity to recombinant SAG1. Despite being N-glycosylated, recombinant SAG1 was recognized by reagents specific for native SAG1. We wished to determine whether our recombinant protein might prove to be a useful diagnostic reagent. Individual human patient sera which had been previously characterized for toxoplasma reactivity, were tested for reactivity to affinity-purified recombinant SAG1, CHO/SAG1, CHO/DHFR, and toxoplasma tachyzoite lysate by Western blot (Fig. 5). All six toxoplasma-seropositive samples, including some obtained from human immunodeficiency virus-positive patients, were reactive with our recombinant SAG1. Even samples with very low toxoplasma titers (1:32 by the Sabin-Feldman dye test, with <1:16 considered negative) were able to recognize the recombinant antigen. Neither of the two negative samples recognized recombinant SAG1. Both negative and positive sera reacted with some proteins in CHO cells, but this did not adversely affect our ability to interpret the immunoblots.

DISCUSSION

We have used CHO cells to produce large amounts of the toxoplasma surface antigen SAG1. This antigen is processed similarly to native SAG1 in that it is apparently correctly folded (as determined by immunoreactivity and the mobility of *N*-glycanase-treated protein in SDS gels) and glycolipid anchored. This indicates that signals for glycolipid addition from lower eukaryotes such as toxoplasma are recognized by mammalian cells, although further analysis will be necessary to determine whether the site of glycolipid addition is identical to that of native SAG1 (which is not known).

Despite being N-glycosylated in CHO cells, recombinant SAG1 is efficiently recognized by antiserum to the native protein and human immune sera. It is unclear why toxoplasma does not use the consensus N-glycosylation site present in SAG1. Although most toxoplasma surface proteins are not glycosylated, one, p23, is N-glycosylated, indicating that the appropriate machinery is present within the secretory pathway of this parasite (19). Although it does not appear to be an obstacle at present, if the addition of N-linked sugars proves to be detrimental, recombinant SAG1 could be expressed in cells that are N-glycosylation deficient (1). Alternatively, site-directed mutagenesis could be used to eliminate the consensus N-glycosylation site.

It is clear that expression of SAG1 within the context of the eukaryotic secretory pathway rather than the reducing environment of the *E. coli* cytosol is important for appropriate folding and disulfide bonding. Fully processed SAG1 contains 12 cysteines, and our work with a SAG1 mutant has revealed that alteration of one cysteine is sufficient to cause incorrect folding (the unreduced mutant protein comigrated with reduced wild-type SAG1) and abolish recognition by a battery of monoclonal antibodies recognizing native or nonreduced SAG1 (14a). In addition, work in collaboration with other investigators has shown that antiserum to a SAG1–glutathione-*S*-transferase fusion protein expressed in baby hamster kidney cells with the Sindbis virus expression system, unlike antiserum made to bacterially expressed fusion protein, recognizes native SAG1 (31).

Further testing of our recombinant antigen is necessary to determine whether it will prove to be sufficiently sensitive and specific for general diagnostic testing, but the high immunogenicity and lack of strain variability of SAG1 make it a very promising candidate. Previous comparison of the deduced amino acid sequences of the virulent strain RH and the nonvirulent strains PLK and CEP indicated that SAG1 from RH differs at only eight amino acid residues from SAG1 from PLK and CEP (2). Of these, only five changes are likely to be present in mature processed SAG1. (PLK and CEP were identical in their coding region.) Furthermore, restriction fragment length polymorphism analysis of the *SAG1* locus of 26 strains gathered from around the world indicated that the *SAG1* locus is apparently represented by only two alleles within toxoplasma strains (26). We know of no reagents or circumstances that will distinguish SAG1 expressed by different strains.

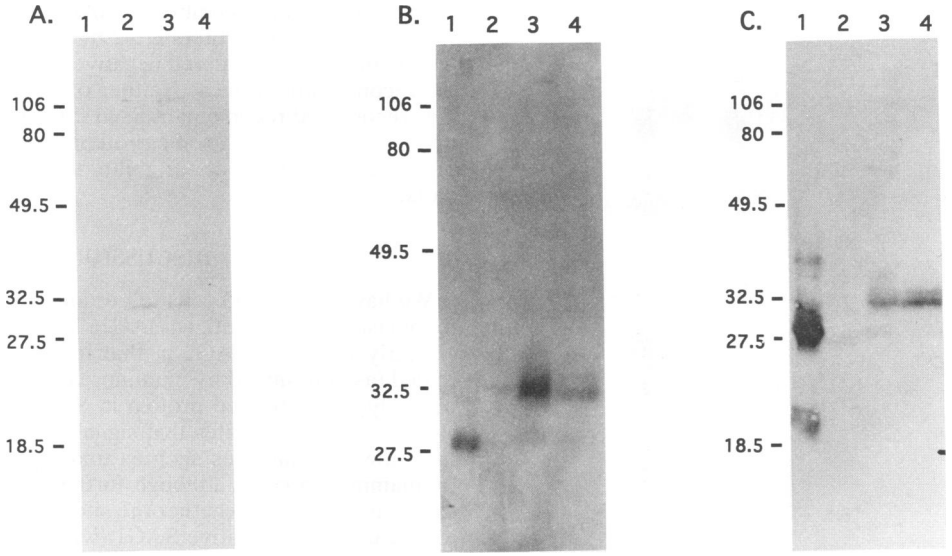

FIG. 5. Reactivity of human immune sera to recombinant SAG1. Individual human serum samples previously tested by the dye test were tested for reactivity to (lanes 1) toxoplasma lysate, (lanes 2) CHO/DHFR cells, (lanes 3) CHO/SAG1 cells, and (lanes 4) affinity-purified recombinant SAG1. Titers by the dye test were (A) negative, (B) 1:128, and (C) 1:1,028.

It will be particularly important to determine whether IgM and IgA produced in response to acute infection react with our recombinant protein. A recent study evaluating 195 human serum samples for IgM and IgA reactivity to SAG1 by immunoblotting showed that 95.5% of the reactive sera recognized SAG1 from both of the two strains used as sources of antigen (one was RH, the source of our recombinant SAG1 gene) (9). We and others (10) have consistently noted that immune sera from infected animals and humans detect SAG1 with significantly less efficiency when reactivity is tested on reduced gels. This group used reduced samples for their analyses, and this may partially explain why not all human samples recognized SAG1 from both strains.

The combination of mammalian expression and FACS selection of cells expressing large amounts of SAG1 on their surface should allow the routine production of large quantities of biologically relevant antigen. This will facilitate its prospects as a component of a subunit vaccine and is clearly an approach which should be useful for the production of other parasite surface antigens which are being evaluated as vaccine candidates. Furthermore, recombinant SAG1 should also prove valuable in dissecting the biological role of this protein and determining what its exact role is in parasite invasion (8, 17).

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