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Characterization of an Immunoprotective Protein Complex of *Anaplasma marginale* by Cloning and Expression of the Gene Coding for Polypeptide Am105L

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Immunization with an *Anaplasma marginale* surface protein complex containing two polypeptides (Am105U and Am105L), each having a molecular weight of 105,000, protected cattle against challenge with virulent organisms. These polypeptides were immunoprecipitated together from detergent extracts of *A. marginale* by a neutralizing monoclonal antibody. After surface radioiodination of intact parasites, both Am105U and Am105L contained the radiolabel. To define the structural and antigenic relationships between Am105U and Am105L and to determine individual efficacies as protective immunogens, we cloned and expressed *A. marginale* DNA in *Escherichia coli*. We identified recombinant bacteria which expressed a novel protein of 105,000 molecular weight as a major cellular component. The recombinant protein was structurally and antigenically homologous to Am105L. There were multiple, partially homologous copies of the cloned DNA sequence in the rickettsial genome.

Hemoparasitic diseases remain endemic in one-half of the world's livestock production areas and are the most severe constraint on improved meat, milk, and fiber production in developing countries (20). The most prevalent of these diseases, anaplasmosis, occurs throughout tropical and subtropical regions and is responsible for 50,000 to 100,000 cattle deaths annually in the United States, which has few endemic areas (7, 12). Despite the impact of the disease, effective immunoprophylaxis has not been developed against the causative rickettsia, *Anaplasma marginale*. This lack of progress results from the complexity of the organism, including persistence in the host and presence of antigenically variant strains.

Our approach to development of a more effective vaccine is to identify one or more surface proteins that are common to several isolates and capable of inducing protection in immunized cattle. We have previously demonstrated that polyclonal antibodies directed against the surface of *A. marginale* initial bodies are able to neutralize infectivity (19). Subsequent data have shown that the neutralizing monoclonal antibodies $15D_2$ and $22B_1$ immunoprecipitate a surface protein of 105,000 molecular weight from radiolabeled *A. marginale* (17). Immunization with this immunoaffinity-isolated protein, termed Am105, protects cattle against challenge with virulent *A. marginale* (17). These data indicate that vaccination of cattle against anaplasmosis is feasible and that Am105 is a candidate immunogen.

In this report, we demonstrate that Am105 consists of a complex of two noncovalently linked polypeptides of similar molecular weight. To determine whether these two polypeptides, termed Am105U and Am105L, are products of separate genes, and to examine the structural and antigenic relationships between the polypeptides, we cloned and expressed genes coding for Am105L epitopes in *Escherichia coli*. In this report, we identify Am105U and Am105L as separate gene products, each bearing surface-exposed epitopes. Cloning and expression of Am105L will allow determination of its efficacy as a single, noncomplexed immunogen.

MATERIALS AND METHODS

Preparation of antisera. Mouse monoclonal antibodies were prepared as described before (14, 17) and designated as follows: $1E_1$ and $24A_1$, control antibodies to a surface glycoprotein of *Trypanosoma brucei*; $F19E_1$, an antibody that immunoprecipitates Am36 (19); $15D_2$ and $22B_1$, antibodies that immunoprecipitate Am105 and neutralize infectivity of *A. marginale* in vitro (17); and $F34C_1$, an antibody that immunoprecipitates Am105.

Antisera to Am105 (17), to isolated *A. marginale* initial bodies (19), and to *E. coli* containing pBR322 or pAM25 plasmid DNA were made in rabbits. Rabbits were immunized four times with lysed bacteria (2×10^9 organisms in complete Freund adjuvant for the first immunization, and 10^{10} organisms in incomplete adjuvant for the other three). Titers were evaluated by an enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (3), or immunoprecipitation of [^{35}S]methionine-labeled extracts of *A. marginale* (2). These rabbit antisera are designated as follows: R612, a control antibody prepared against a surface glycoprotein of *T. brucei*; R781, an antibody prepared against isolated initial bodies of *A. marginale*; R873 and R874, antibodies prepared against Am105 isolated by immunoaffinity chromatography on monoclonal antibody-Sepharose 4B (17) (purified Am105 consists of Am105U and Am105L); R907, an antibody prepared against *E. coli*(pBR322); and R911, an antibody prepared against *E. coli*(pAM25).

Antigen detection on nitrocellulose filters. Proteins of *A. marginale* or recombinant *E. coli* were bound to nitrocellulose filters and detected by reaction with specific antisera and ^{125}I-labeled protein A as described by Young and Davis

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(26), with two modifications: (i) after chloroform lysis, filters were fixed in 10% acetic acid–25% isopropanol; and (ii) 1% hemoglobin was added to buffers instead of bovine serum albumin to block nonspecific binding of ^{125}I-labeled protein A to the filters.

ELISA. ELISAs were as described by Ellens and Gielkens (6), using Am105 attached to plates at 50 ng per well. The enzyme label was horseradish peroxidase-protein A, and the substrate was recrystallized 5-aminosalicylic acid. Am105 was isolated from *A. marginale* by immunoaffinity chromatography on monoclonal antibody 15D$_2$-Sepharose 4B (17) and consisted of Am105U and Am105L. Sera against Am105 and against *E. coli* containing pBR322 or pAM25 were prepared in rabbits.

Immunoprecipitation. *A. marginale* organisms were radiolabeled by metabolic incorporation of [^{35}S]methionine during short-term in vitro culture (2) or by surface radioiodination, using lactoperoxidase (19). *E. coli* organisms were also labeled with ^{35}S during exponential growth in 1-ml cultures containing 250 μCi of [^{35}S]methionine and 35 μg of ampicillin per ml. After removal of the unincorporated radiolabel, organisms were solubilized by sonication at 4°C in a lysis buffer consisting of 50 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) Nonidet P-40. The solubilized extract was centrifuged at 130,000 × *g* for 1 h at 4°C and passed through a 0.2-μm-pore-size filter (Centrex; Schleicher & Schuell, Inc.) before being used for immunoprecipitation with rabbit or mouse antibodies and protein A-bearing *Staphylococcus aureus* (Calbiochem) (9, 17, 23). The precipitated radiolabel was eluted and analyzed on 7.5 to 17.5% polyacrylamide–SDS gels, 7.5% polyacrylamide gels containing 4 M urea, or 5% polyacrylamide gels containing 4 M urea. ^{14}C-labeled standard proteins were as follows (molecular weight): myosin (200,000), phosphorylase *b* (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

For the experiment described below (see Fig. 5), immunoprecipitated recombinant Am105, Am105U, and Am105L protein bands were cut out from dried 7.5% polyacrylamide–4 M urea gels and then separately rehydrated and electroeluted into a mixture of 50 mM Tris hydrochloride (pH 8.0), 0.1% (wt/vol) SDS, and 1% (vol/vol) Nonidet P-40. Polyacrylamide was removed by centrifugation, and the ^{35}S-labeled proteins were immunoprecipitated again from electroelution buffer.

Peptide mapping. Immunoprecipitated, ^{35}S-labeled proteins were cut out from dried polyacrylamide gels and compared for sequence homology by peptide mapping as described before (5). Radiolabeled peptides produced by limited proteolysis with *S. aureus* V8 protease were separated on 15% polyacrylamide–SDS gels and detected by fluorography (4).

Isolation of *A. marginale* DNA. Bovine blood, infected with *A. marginale* at >50% erythrocytic parasitemia, was washed four times with phosphate-buffered saline. At each wash an upper layer containing leukocytes and erythrocytes was removed. The remaining erythrocytes were then frozen in phosphate-buffered saline at a packed cell volume of 50%. A 100-ml volume of the erythrocyte suspension was thawed and centrifuged at 30,000 × *g* for 20 min at 4°C to pellet *A. marginale* initial bodies and erythrocyte membranes. The pellet was washed a further three times in phosphate-buffered saline at 30,000 × *g* to remove hemoglobin from the lysed erythrocytes. DNA was then extracted from initial bodies (11) and further purified by deproteinization with phenol-chloroform, digestion with RNase A and proteinase K, and precipitation with ethanol.

Preparation of recombinant DNA libraries. *A. marginale* DNA was partially digested with *Sau*3A to an average size of 5 kilobases (kb). Digested DNA was ligated with *Bam*HI-cleaved and dephosphorylated pBR322, using T4 DNA ligase (25). *E. coli* HB101 cells were transformed to ampicillin resistance by the high-efficiency transformation protocol of Hanahan (8). Plasmids pAM22 and pAM25 were identified by expression screening of a library containing 8,000 recombinants with R873 serum (rabbit anti-[Am105U plus Am105L] complex). Other colonies in this library, such as that containing pAM14, also reacted with R873 and contained the pAM22 sequence plus various lengths of additional DNA that extended beyond the *Bgl*II sites.

A second library of 3,000 recombinants was prepared by digesting *A. marginale* DNA to completion with *Bgl*II and ligating into the *Bam*HI site of pBR322. Clones containing pAM97 and pAM113 were identified in this library by expression screening with R873.

Southern blotting. The protocol used was a modification of that described by Wahl et al. (24). Portions (0.5 μg) of *A. marginale* genomic DNA or plasmid DNA (0.36 μg) were digested with the appropriate restriction enzymes. For comparison of plasmid and genomic sequences on the same gel, 0.5 μg of digested genomic DNA or 1.8 ng of plasmid DNA was subjected to agarose gel electrophoresis and blotted onto nitrocellulose filters. Hybridization was at 65°C in 5× SSPE (0.18 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA [pH 7.4])–0.25% Sarkosyl (Sigma) containing 10% dextran sulfate, 100 μg of denatured calf thymus DNA per ml, and a ^{32}P-labeled nick-translated probe. Filters were washed a total of five times, finally in 0.1× SSPE–0.0033% Sarkosyl at 65°C. The probe was the 1.4-kb *Hin*cII-*Hin*dIII fragment of pAM14 or the 2.0-kb *Sst*I fragment of pAM97, isolated from agarose gels.

RESULTS

Genomic libraries and Am105 expression by *E. coli*. Initial experiments investigated the specificity and sensitivity of immunoblot assays in detecting *A. marginale* proteins immobilized on nitrocellulose filters (26). In previous studies we prepared monoclonal and polyvalent antisera against *A. marginale* which had specificity for different surface proteins (17–19). The reactions of these antisera with positive and negative control antigens are shown in Fig. 1A. All antibodies detected *A. marginale*-infected erythrocytes and did not react with noninfected erythrocytes. The sensitivity of detection was greatest with R873, a rabbit antiserum against immunoaffinity-isolated Am105. R873 detected as few as 1,200 parasitized erythrocytes in the 1-μl spot applied to the filter. The specificity of each antibody in immunoblots was the same as that observed previously in immunoprecipitation experiments. Polyvalent or monoclonal antibodies against Am105 or another surface protein, Am36, reacted with the appropriate protein; there were no cross-reactions or reactions with the negative control, ovalbumin. R873 detected a minimum of 1 ng of purified Am105. R781 was an antiserum prepared against isolated *A. marginale* initial bodies; it immunoprecipitated both Am105 (Am105U and Am105L) and Am36 (data not shown), and recognized Am105 and Am36 in immunoblots (Fig. 1A). We thus considered this assay sufficiently sensitive and specific to detect expression of *A. marginale* proteins in recombinant *E. coli*.

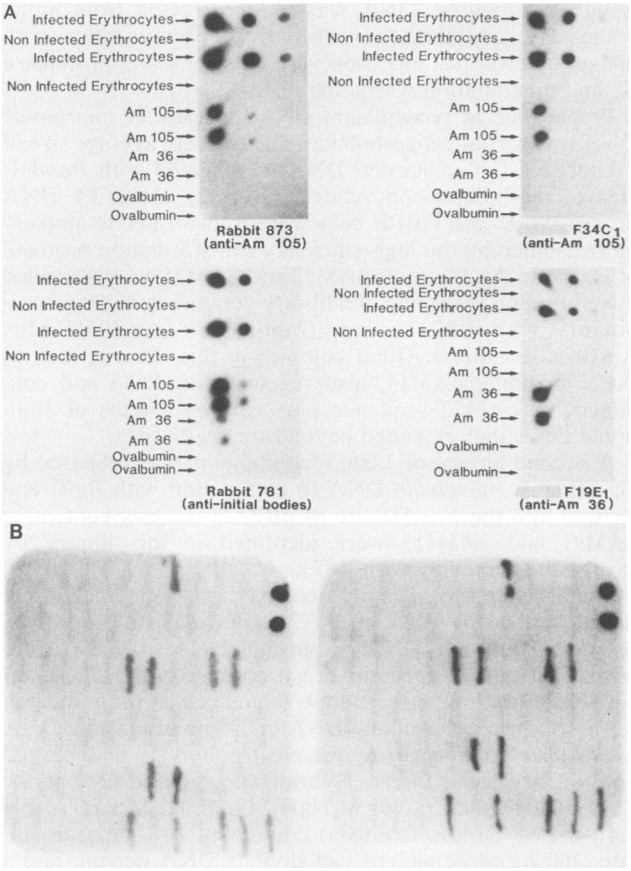

FIG. 1. Detection of *A. marginale* proteins on nitrocellulose with antibody and ^{125}I-labeled protein A. (A) Known positive and negative control antigens were applied to all filters in sequential 10-fold dilutions and in duplicate: *A. marginale*-infected erythrocytes (2×10^5 to 2×10^3 total cells at 60% parasitemia) and noninfected erythrocytes (same concentration), Am105 protein (10 to 0.1 ng), Am36 protein (10 to 0.1 ng), and ovalbumin (10 to 0.1 ng). A different antibody was tested on each filter: R873 (1:4,000 dilution), R781 (1:400 dilution), $F34C_1$ (2 μg/ml), and $F19E_1$ (2 μg/ml). (B) Recombinant *E. coli*, selected as potentially positive colonies in a previous screen, were rescreened on duplicate filters for reaction with R873. The two spots at the top right of each filter are duplicate signals from a positive control antigen: 1 μl containing 2×10^4 total erythrocytes at 60% parasitemia. Uninfected erythrocytes (1 μl) were also applied in duplicate to each filter and gave no signal.

Previous data have suggested that gene regulatory sequences of rickettsiae may function in *E. coli* (10, 13, 25). Accordingly, parasite DNA was extracted from bovine erythrocytes containing a Florida isolate of *A. marginale*. The DNA was partially digested with *Sau*3A, inserted into the *Bam*HI site of phosphatase-treated pBR322, and used to transform *E. coli* HB101 to ampicillin resistance. This genomic library was screened with R873 in the immunoblot assay for expression of Am105 antigenic determinants.

E. coli colonies containing recombinant plasmids of various sizes reacted stably with the antiserum (Fig. 1B). The restriction enzyme maps of insert DNAs from pAM22 and pAM25, the smallest plasmids of expressing colonies (3.75 and 4.15 kb, respectively), are shown in Fig. 2. All plasmids from expressing bacteria contained the inserted sequence present in pAM22; there were various lengths of additional insert DNA in the larger plasmids which extended beyond the *Bgl*II sites. Restriction enzyme mapping and Southern blotting suggested that the shaded sequence of 240 base pairs in pAM25 was not contiguous with the remainder of pAM25 DNA in the *A. marginale* genome and that two *Sau*3A fragments were ligated in this plasmid during cloning. Both possible insert orientations with respect to pBR322 DNA were found in plasmids from expressing colonies (Fig. 2).

Analysis of each expressed plasmid DNA, and of genomic DNA by Southern blotting, suggested that the inserted sequence in pAM22 should be contained within a single *Bgl*II fragment of *A. marginale* genomic DNA. To confirm this, we prepared a second library. *A. marginale* DNA was digested to completion with *Bgl*II and inserted into the *Bam*HI site of pBR322. Plasmids pAM97 and pAM113 were identified in this library by expression screening with R873; they contained the expected *Bgl*II fragment in both orientations (Fig. 2).

Proteins expressed by recombinant *E. coli*. To characterize novel proteins synthesized by recombinant *E. coli*, bacteria containing either pAM25 or pBR322 were radiolabeled by metabolic incorporation of [^{35}S]methionine. The radiolabeled proteins were analyzed by immunoprecipitation and SDS gel electrophoresis. The protein profile of recombinant *E. coli* is shown in Fig. 3, lane 7, and may be compared with the analogous profile of control bacteria containing only pBR322 (lane 8). All protein bands were present in both lanes, except for a major radiolabeled polypeptide of 105,000 molecular weight in recombinant bacteria. When labeled proteins were immunoprecipitated by R873, one normal *E. coli* protein was recognized. However, in recombinant bacteria, the additional 105,000-molecular-weight protein was also precipitated (compare lanes 5 and 10). A similar result was obtained with a different antiserum to Am105, R874 (lanes 3 and 12). These results demonstrated that a novel protein, coded for by pAM25 DNA, was expressed as a major component of the recombinant bacteria. This protein had a similar molecular weight and shared antigenic determinants with immunoaffinity-isolated Am105 from *A. marginale*.

R873 and R874 reacted with one or two normal *E. coli* proteins when used undiluted in immunoprecipitation, presumably because of prior exposure of rabbits to the bacterium. The possibility of a cross-reaction between Am105 and *E. coli* proteins is considered less likely, because antisera to lysed nonrecombinant *E. coli* did not recognize Am105 (see Fig. 5 and 6). The reaction of R873 with *E. coli* was not observed in immunoblot assays because the dilution of antiserum used (1:4,000) effectively removed anti-*E. coli* activity while retaining activity against Am105.

The molecular weight of the recombinant protein was identical in bacteria containing pAM25, pAM22, pAM97, or pAM113 plasmids. The level of expression in each of these recombinants was also comparable, as judged by relative band intensity on SDS gels. The orientation of insert DNA with respect to pBR322 had no apparent effect on expression (both orientations were equally represented in the four plasmids). These data suggest the following: (i) that the *A. marginale* gene is functional in *E. coli*; (ii) that the gene is contained within the cloned *Bgl*II fragment; and (iii) that the expressed molecule is not a fusion protein composed of both pBR322- and *A. marginale*-encoded amino acids.

Recombinant Am105 is structurally homologous to nonrecombinant Am105L. Recombinant Am105 was recognized by R873 and hence was antigenically homologous with Am105U and/or Am105L polypeptides. However, recombinant Am105, expressed by any of the recombinants, was not recognized by monoclonal antibodies $22B_1$ or $15D_2$ in immu-

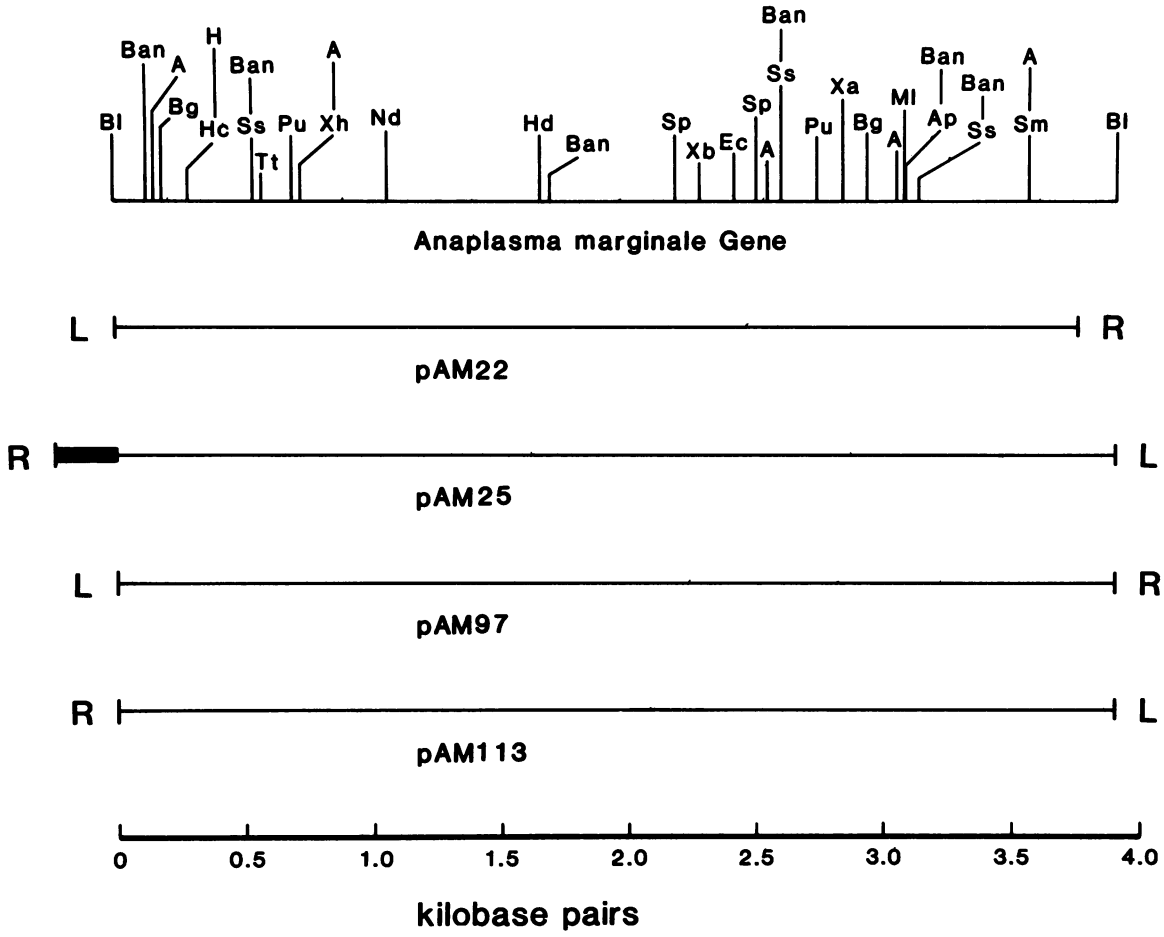

FIG. 2. Restriction enzyme maps of plasmid insert DNA from *E. coli* colonies expressing Am105 determinants. L (left) and R (right) refer to the orientation of insert DNA with respect to pBR322 sequences. L is proximal to the pBR322 *Eco*RV site, and R is proximal to the *Sph*I site. A, *Ava*I; Ap, *Apa*I; Ban, *Ban*II; Bg, *Bgl*I; Bl, *Bgl*II; Ec, *Eco*RV; H, *Hpa*I; Hc, *Hin*cII; Hd, *Hin*dIII; Ml, *Mlu*I; Nd, *Nde*I; Pu, *Pvu*II; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; Tt, *Tth*111I; Xa, *Xma*III; Xb, *Xba*I; Xh, *Xho*I.

noprecipitation or immunoblot assays (data not shown), or by R781 (Fig. 3, lanes 2 and 13). There were, therefore, important antigenic differences between recombinant and native Am105. We compared recombinant Am105 for structural homology with each component of the Am105 doublet, Am105L and Am105U. *A. marginale* was radiolabeled with [^{35}S]methionine, solubilized, and immunoprecipitated with the neutralizing monoclonal antibody $22B_1$, and the precipitated proteins were separated by electrophoresis in a 7.5% polyacrylamide–SDS gel containing 4 M urea (Fig. 4A, lane 3). The Am105 doublet was clearly resolved. No bands were visible in the control lane (*A. marginale* plus $24A_1$ monoclonal antibody, lane 4). Recombinant Am105, immunoprecipitated by R873, was analyzed on the same gel. The recombinant Am105 migrated as a single band in an identical position to Am105L (Fig. 4A, lane 1).

The Am105 doublet in this gel system was resolved sufficiently to allow cutting out of the Am105L and Am105U components of the immunoprecipitate from a dried gel. Gel fragments containing each polypeptide were then rehydrated and analyzed by peptide mapping (5). Recombinant Am105, immunoprecipitated by R873, was also cut out and analyzed.

Figure 4B shows a peptide map obtained by partial digestion of the eluted polypeptides with *S. aureus* V8 protease. Cleavage peptides of recombinant Am105 closely resembled those of Am105L. Initial proteolysis products of both recombinant Am105 and Am105L were polypeptides of 75,000, 59,000, and 51,500 molecular weight. Identical low-molecular-weight components (34,300, 18,600, and 13,000 to 16,000) were also generated. Therefore, the recombinant Am105 and Am105L molecules were homologous and possibly identical.

In contrast, cleavage peptides produced from Am105U were largely dissimilar to both Am105L and recombinant Am105. Predominant digestion products of Am105U in the 22,000- to 27,000-molecular-weight range had no counterpart in Am105L or recombinant Am105. Another peptide of 16,000 molecular weight was also absent from Am105L and recombinant Am105. Although different peptides were generated from Am105L and Am105U by proteolysis, the sensitivity of this procedure did not permit a determination of total nonhomology between Am105L and Am105U. For example, cleavage peptides of 29,500 were produced from both Am105L and Am105U. Whether these two low-molecular-weight peptides share homology will require further structural analysis.

Antigenic relationships among recombinant Am105, Am 105L, and Am105U polypeptides. The antigenic relationships among Am105L, Am105U, and recombinant Am105 were investigated by preparing antisera against bacteria express-

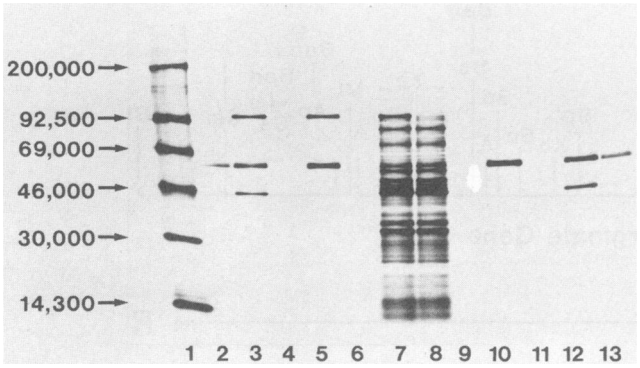

FIG. 3. *A. marginale* proteins synthesized by recombinant *E. coli*. *E. coli* organisms containing pBR322 or pAM25 plasmid DNA were radiolabeled with [^{35}S]methionine during in vitro culture, and a detergent extract was immunoprecipitated with different antisera. Immunoprecipitates were analyzed by 7.5 to 17.5% polyacrylamide-SDS gel electrophoresis and fluorography. Lanes: 1, ^{14}C-labeled molecular weight standard proteins; 2 to 7, *E. coli* plus pAM25; 8 to 13, *E. coli* plus pBR322; 7 and 8, total ^{35}S-protein profiles. Immunoprecipitating antibodies were normal rabbit serum (lanes 6 and 9), R873 (lanes 5 and 10), R612 (lanes 4 and 11), R874 (lanes 3 and 12), and R781 (lanes 2 and 13).

ing recombinant Am105 in four rabbits; another four rabbits were immunized with *E. coli* containing pBR322 as a control. Sera were tested for recognition of nonrecombinant Am105 by an ELISA. All rabbits immunized with recombinant bacteria developed antibodies to Am105, ranging in titer from 1:100 to 1:1,000. No rabbits immunized with control *E. coli* developed antibodies to Am105. The anti-recombinant-Am105 sera immunoprecipitated both Am105L and Am105U from [^{35}S]methionine-labeled *A. marginale* (data not shown), and therefore reacted similarly to R873 and 22B$_1$ antibodies.

There are two possible explanations for these results. First, Am105L and Am105U may share antigenic determinants and therefore be immunoprecipitated together. Second, Am105L and Am105U may be antigenically unrelated but complexed. To discriminate between these possibilities, Am105L and Am105U were separately purified and immunoprecipitated. A detergent extract of [^{35}S]methionine-labeled *A. marginale* was first immunoprecipitated with monoclonal antibody 22B$_1$, and the Am105L and Am105U components of the precipitate were separated by SDS gel electrophoresis. The Am105L and Am105U bands were cut out, electroeluted, and then separately immunoprecipitated again with monoclonal antibody 22B$_1$ or with rabbit anti-recombinant-Am105 serum (Fig. 5). Only Am105U was reimmunoprecipitated by 22B$_1$; Am105L was not recognized (lanes 4 and 5). In contrast, anti-recombinant-Am105 serum immunoprecipitated Am105L but not Am105U (lanes 8 and 9) when the two components were separated before immunoprecipitation. Therefore, recombinant Am105 was antigenically homologous only to Am105L.

Thus, Am105 exists as a complex of two polypeptides, Am105L and Am105U. Monoclonal antibody 22B$_1$ recognizes an epitope present on Am105U, and binding to that epitope causes precipitation of both components of the complex. The complex is stable in 1% Nonidet P-40 and 0.1% SDS, which are present in the immunoprecipitation reaction, but is dissociated by boiling in SDS gel sample buffer. Am105L and Am105U are apparently not linked by

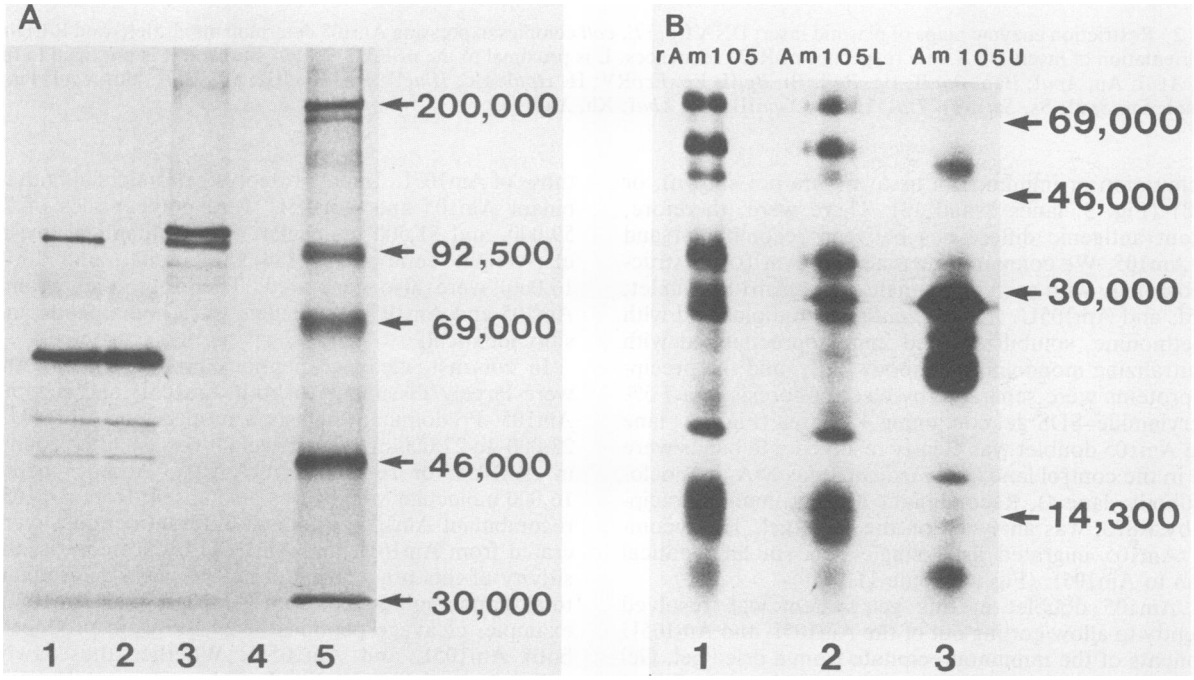

FIG. 4. Comparison of recombinant Am105 (rAm105) with Am105L and Am105U. (A) *E. coli* cells containing pAM25 (lane 1) or pBR322 (lane 2) were radiolabeled with [^{35}S]methionine during in vitro culture, and a detergent extract was immunoprecipitated with R873. *A. marginale* was also labeled with [^{35}S]methionine and immunoprecipitated with neutralizing monoclonal antibody 22B$_1$ (lane 3) or with control monoclonal antibody 24A$_1$ (lane 4). Immunoprecipitates were analyzed on a 7.5% polyacrylamide–SDS gel containing 4 M urea; lane 5, ^{14}C-labeled molecular weight standard proteins. (B) Partial proteolysis products of recombinant Am105, Am105L, and Am105U, produced by digestion in the stacking gel with 0.025 μg of *S. aureus* V8 protease, were compared on a 15% polyacrylamide–SDS gel.

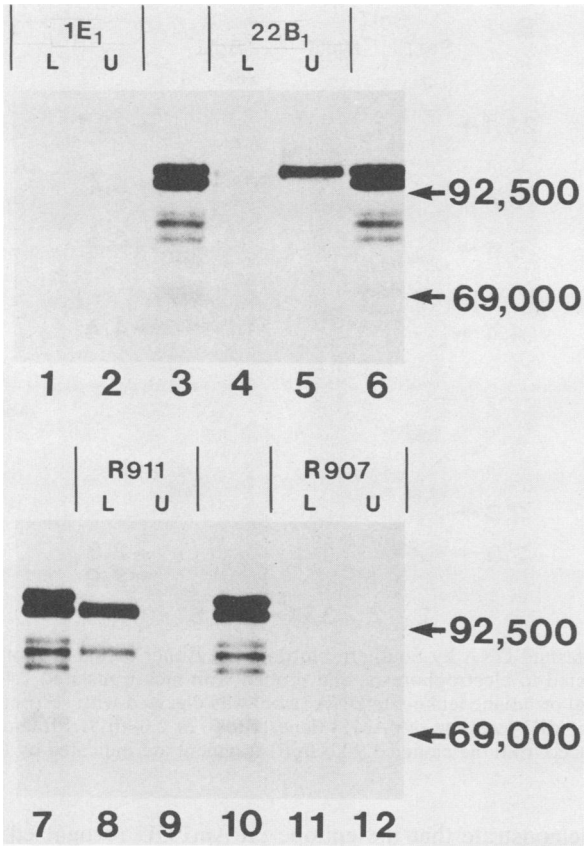

FIG. 5. Antigenic comparison of recombinant Am105, Am105U, and Am105L. ^{35}S-labeled Am105U and Am105L were immunoprecipitated, separately or together, with different antibodies as indicated. All precipitates were analyzed on 7.5% polyacrylamide–SDS gels containing 4 M urea: Am105L, lanes 1, 4, 8, and 11; Am105U, lanes 2, 5, 9, and 12; and both Am105U and Am105L ($22B_1$ precipitates of ^{35}S-labeled *A. marginale*), lanes 3, 6, 7, and 10.

disulfide bonds, because the molecular weight is unchanged when electrophoresis is performed under reducing or nonreducing conditions. Recombinant Am105 is structurally and antigenically homologous to Am105L. No evidence was obtained for structural or antigenic homology between recombinant Am105 and Am105U polypeptides or between Am105L and Am105U. These data explain the positive reaction of recombinant Am105 with rabbit anti-Am105 sera and a negative reaction with monoclonal antibody $22B_1$.

Surface radiolabeling of *A. marginale* initial bodies labels both Am105L and Am105U. Viable initial bodies were radiolabeled with ^{125}I, using lactoperoxidase as described before (19). Labeled extracts were then immunoprecipitated with R911 (anti-recombinant Am105), R873 (anti-Am105), monoclonal antibody $22B_1$, or the appropriate control antibody. The precipitates were analyzed on polyacrylamide gels containing 4 M urea (Fig. 6). The results showed that both Am105L and Am105U polypeptides contained the radiolabel and were precipitated by R911, R873, and $22B_1$. The increased band intensity of Am105U when precipitated by $22B_1$ and of Am105L when precipitated by R911 suggests some dissociation of the Am105L-Am105U complex during this immunoprecipitation.

***A. marginale* genome contains multiple copies of the cloned *Bgl*II fragment.** *A. marginale* genomic DNA was cut with restriction enzymes; the DNA fragments were separated by gel electrophoresis, blotted to nitrocellulose, and probed with ^{32}P-labeled plasmid insert DNA from bacteria expressing recombinant Am105. By using enzymes which did not cut within the probe sequence, we observed multiple hybridizing bands (Fig. 7A, lanes 7 and 8). To discover whether these represented partially homologous copies of the cloned sequence or polymorphism in flanking regions, we cleaved genomic DNA with restriction enzymes that would generate a predictable fragment. *Hin*cII plus *Mlu*I digestion should yield a 2.8-kb fragment hybridizing to the *Hin*cII-*Hin*dIII probe. For comparison, plasmid DNA containing the entire 3.9-kb *Bgl*II fragment was also digested with *Hin*cII plus *Mlu*I and analyzed in the adjacent gel lane (Fig. 7A, lanes 5 and 6). The expected 2.8-kb fragment was found in both digests, but hybridizing bands of 4.0 and 6.7 kb were also observed in the genomic DNA. The 4.0- and 6.7-kb bands must represent partially homologous copies of the 3.9-kb cloned *Bgl*II fragment that do not have the *Hin*cII or *Mlu*I site or both. Similar digests with *Hin*cII plus *Bgl*II or *Sst*I, *Bgl*I, or *Bgl*II alone always produced the DNA fragment expected from the map in Fig. 2, but with between two and four additional hybridizing bands (Fig. 7A and B). Multiple hybridizing bands were detected whether the *Hin*cII-*Hin*dIII or *Sst*I probes were used in detection (Fig. 7B, lanes 2 and 5). There was no hybridization between cloned probe and bovine leukocyte DNAs (Fig. 7B, lane 4), further demonstrating the parasite origin of the cloned sequence.

Thus, the cloned DNA faithfully represents an *A. marginale* genomic sequence. However, additional partially homologous copies of the cloned 3.9-kb *Bgl*II fragment are also present in the genome.

DISCUSSION

The data presented describe the expression of an *A. marginale* protein of 105,000 molecular weight in recombinant *E. coli*. Antisera prepared in rabbits against immunoaffinity-isolated, nonrecombinant Am105 recognize recombinant Am105 and vice versa, showing shared epitopes. Also, antisera against recombinant Am105 react with *A. marginale* in immunofluorescence and agglutinate purified initial bodies, demonstrating the presence of recombinant Am105 epitopes on the parasites themselves. Recombinant Am105 is structurally and antigenically homologous to Am105L; no evidence was obtained for homology to Am105U.

Nonrecombinant Am105, containing both Am105L and Am105U, confers protection on cattle against challenge with

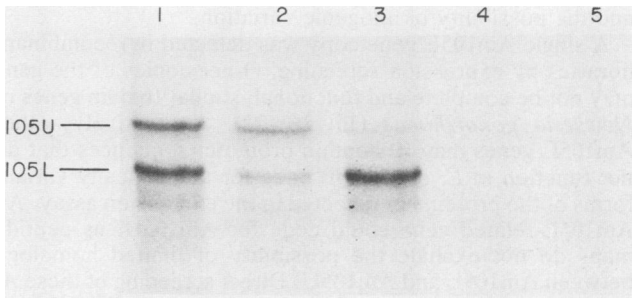

FIG. 6. Surface radiolabeling and immunoprecipitation of *A. marginale* initial bodies. Initial bodies were radiolabeled with ^{125}I, using lactoperoxidase, and a detergent extract was immunoprecipitated with R873 (lane 1), monoclonal antibody $22B_1$ (lane 2), R911 (lane 3), monoclonal antibody $1E_1$ (lane 4), and R907 (lane 5). Immunoprecipitates were analyzed on a 5% polyacrylamide–SDS gel containing 4 M urea.

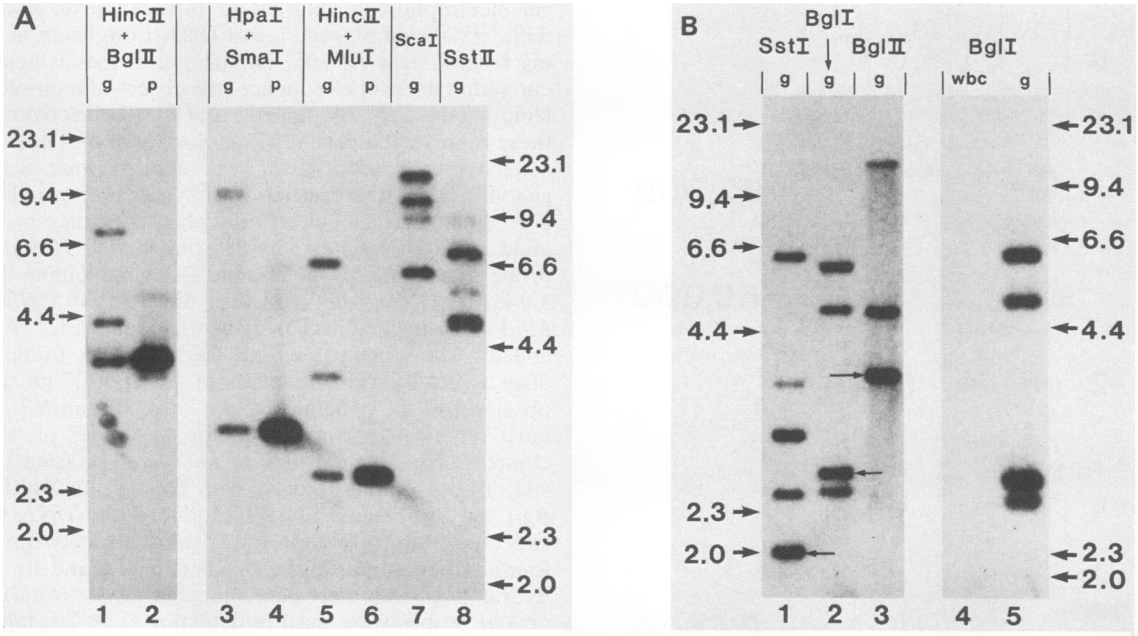

FIG. 7. Comparison of *A. marginale* genomic DNA with recombinant plasmid DNA by Southern blotting. (A) Either pAM14 (p) or *A. marginale* genomic DNA (g) was digested with restriction enzymes, subjected to electrophoresis, and probed with nick-translated 1.4-kb *Hin*cII-*Hin*dIII insert DNA from pAM14. (B) *A. marginale* genomic DNA (g) or bovine leukocyte DNA (wbc) was digested with restriction enzymes, subjected to electrophoresis, and probed with the 1.4-kb *Hin*cII-*Hin*dIII fragment of pAM14 (lanes 1 to 3) or 2.0-kb *Sst*I fragment of pAM97 (lanes 4 and 5). The genomic bands corresponding to those produced from the cloned 3.9-kb *Bgl*II fragment are indicated by thin arrows on the gels.

A. marginale (17). It is not known whether Am105L or Am105U, used separately as an immunogen, would confer protection. Am105L and Am105U are both accessible on viable initial bodies to surface radiolabeling, one important criterion for an immunoprotective protein (1). Am105U may be more likely to induce protection because this polypeptide contains the epitope recognized by neutralizing monoclonal antibody $22B_1$ (Fig. 5). However, other neutralization-sensitive epitopes may also be present in Am105L. The epitope recognized in Am105U by antibody $22B_1$ is conserved in eight geographically distinct isolates (17), an important practical concern for potential immunization. Rabbit anti-recombinant-Am105 sera also reacted with all isolates tested in immunofluorescence, but variation in surface-exposed epitopes might not be revealed by such polyvalent sera. Examination of the *A. marginale* genome by Southern blotting suggests the presence of a family of Am105L genes and the possibility of antigenic variation.

A single Am105L gene copy was detected in recombinant libraries by expression screening. Other copies of the gene may not be complete and functional, similar to pilin genes of *Neisseria gonorrhoeae* (15, 16, 22). Alternatively, other Am105L genes may (i) contain promoter sequences that do not function in *E. coli* or (ii) code for antigenically variant forms of the protein not detected in the expression assay. An Am105L-related gene could code for Am105U, as peptide maps do not exclude the possibility of limited homology between Am105L and Am105U. Direct screening of these *A. marginale* DNA libraries in pBR322 with monoclonal antibodies did not reveal colonies expressing Am105U epitopes.

Experiments in progress examine whether recombinant Am105 will induce protection in cattle against disease and whether Am105U may be expressed in *E. coli* so that both components of the Am105 complex may be tested for protection. Immunoblot experiments and that shown in Fig. 5 demonstrate that the epitope on Am105U recognized by neutralizing monoclonal antibody $22B_1$ is not denatured by solvents such as 2% SDS, 2.5% mercaptoethanol, 10% acetic acid, and 25% isopropanol. Hence, this epitope is relatively resistant to conformational changes compared with, for example, surface-exposed epitopes of *Trypanosoma brucei* (4a). Other data suggest that immunoaffinity-isolated Am105 is not glycosylated and show that the epitope recognized by antibody $22B_1$ is protease sensitive (G. H. Palmer, S. D. Waghela, W. C. Davis, A. F. Barbet, and T. C. McGuire, Int. J. Parasitol., in press). Expression of the Am105U neutralization-sensitive epitope should, therefore, be readily obtained by direct monoclonal antibody screening of a fusion protein expression library, e.g., in bacteriophage λgt11 (26). In those libraries, expression of Am105U epitopes would not depend on recognition of rickettsial regulatory DNA sequences by *E. coli* (21).

The most effective vaccine against *A. marginale* may be a combination of surface proteins. These include Am86, Am61, Am36, and Am31 as well as Am105 (19). We described here the cloning and expression of one *A. marginale* gene in *E. coli* and structural and antigenic homology between the cloned and native surface proteins. Since cattle are protected against *A. marginale* by immunization with Am105 purified from infected erythrocytes (19), these results suggest that a recombinant vaccine is feasible and provide a rational basis for its development.

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