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Expression and Immune Recognition of the Conserved MSP4 Outer Membrane Protein of *Anaplasma marginale*†

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Defining conserved, protective epitopes is essential to the design of an effective vaccine against bovine anaplasmosis. MSP4, one of six initial body proteins recognized by a neutralizing serum, is conserved among *Anaplasma marginale* isolates at both the protein and the DNA levels. Sera from cattle immunized with an outer membrane fraction of *A. marginale* and protected from a virulent challenge bind MSP4. The gene for MSP4 has been cloned, and the recombinant protein has been expressed, isolated, and demonstrated to share epitopes with the native protein expressed on initial bodies. MSP4 may have a greater potential to protect cattle from a challenge by heterologous isolates than other *A. marginale* surface proteins, which vary widely in size and structure.

Despite extensive losses to bovine anaplasmosis throughout the world's tropical regions (36), there currently is not an effective vaccine against the causative rickettsia, *Anaplasma marginale* (26). Protection against clinical disease by killed organisms or attenuated strains is possible, but variation in the ability to provide cross-protective immunity against heterologous isolates limits the usefulness of these vaccines (20). Current strategies focus on the identification and expression of surface proteins of *A. marginale* initial bodies as the basis for a subunit vaccine. Of six major proteins previously identified with a neutralizing rabbit serum (31), two can induce at least partial protection against clinical disease in cattle (29, 32). However, both of these proteins are encoded by members of multigene families and appear to vary antigenically and structurally among *A. marginale* isolates (28, 28a, 32). Identification of highly conserved proteins and protective epitopes may be critical for a successful vaccine strategy.

Recent work immunizing cattle with fractionated *A. marginale* membranes suggested that outer and inner initial body membrane fractions can protect cattle from a virulent challenge (42). Interestingly, the only protein apparently conserved between these two membrane fractions was approximately the same size as the 31-kDa MSP4 protein, one of the six proteins originally recognized by the neutralizing rabbit serum (31). MSP4, if present in both membrane fractions, could be the basis for this protective immunity.

The MSP4 protein has not yet been investigated as a vaccine candidate. Detection of the *msp4* gene in genomic *A. marginale* libraries by standard hybridization and other screening procedures has been repeatedly unsuccessful; instead, we have originated a method to sequence the gene without prior molecular cloning by use of only the N-terminal protein sequence and the polymerase chain reaction (27). Consequently, the *msp4* gene has been completely sequenced (GenBank accession number L01987) but has not been cloned or expressed, nor have the immunogenic properties of the MSP4 protein been examined. We report here the cloning and direct expression of the recombinant MSP4 protein, the conservation of MSP4 among *A. marginale* isolates at the DNA and protein levels, and the recognition of the recombinant and native MSP4 proteins by immune cattle sera.

MATERIALS AND METHODS

Isolates of *A. marginale*. The following isolates of *A. marginale* were used in these studies: Florida; South Idaho; Missouri; Oklahoma; North Texas; Virginia; Clarkston, Wash.; and Okanogan, Wash. Their isolation and characterization have been described elsewhere (21, 25, 37). All experiments were completed with the virulent Florida isolate unless otherwise noted. Isolates were preserved in liquid nitrogen (22), as frozen packed erythrocytes in PBS (15 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.2]) at −70°C, or as acetone-fixed blood smears at −70°C.

Monoclonal antibodies and polyclonal sera. The following monoclonal antibodies were used in these studies: AnaR76A1, which specifically recognizes MSP4 (42); Ana22B1 and Ana15D2, which recognize *A. marginale* initial body protein MSP1a (29); AnaO58A2, which recognizes *A. marginale* initial body protein MSP2 (32); and Tryp1E1, which recognizes a variable surface glycoprotein of *Trypanosoma brucei*. Polyclonal sera were obtained by immunizing rabbits once with 100 μg of isolated native MSP4 in complete Freund's adjuvant and then three times more at 2-week intervals with MSP4 in incomplete Freund's adjuvant; control rabbits were similarly immunized with native MSP2.

Immunoprecipitations. *A. marginale* organisms were metabolically labeled with [^{35}S]methionine (3) or surface labeled with ^{125}I by use of lactoperoxidase (38). Isolated proteins were dialyzed into 50 mM $(NH_4)_2CO_3$ (pH 8.0) and ^{125}I labeled with Iodobeads (Pierce) for 5 min following manufacturer's instructions. Immunoprecipitations were completed as described previously (30). In brief, lysed cells or isolated proteins were incubated with a monoclonal antibody, and the complexes were precipitated with protein

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G-Sepharose beads (Sigma). The beads were washed six times in 20 mM Tris (pH 7.6)–10 mM NaCl–5 mM EDTA–1% Nonidet P-40–0.1% sodium dodecyl sulfate (SDS) (TEN buffer); the first four washes also contained 2 M NaCl (32). The precipitated proteins were eluted from the beads with protein sample buffer (15% glycerol, 2% SDS, 25 mM Tris [pH 6.8], 2.5% β-mercaptoethanol, 0.002% bromophenol blue) and separated on 7.5 to 17.5% gradient SDS-polyacrylamide gels. ^{35}S-labeled gels were impregnated with En^{3}Hance (NEN), and all gels were dried and exposed to XAR-5 film (Kodak).

Detection of MSP4 among *A. marginale* isolates. *A. marginale* initial bodies were isolated from cryogenically preserves organisms (31) and biotin labeled with sulfosuccinimidobiotin (sulfo-NHS-biotin; Pierce) (13). Following immunoprecipitation (30) with AnaR76A1 or Tryp1E1, the proteins were isolated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin (BSA) in TBS (0.5 M NaCl, 0.02 M Tris [pH 7.5]), incubated with horseradish peroxidase-labeled avidin (1:3,000 in TBS–1% BSA), and detected with 3,3′-diaminobenzidine and hydrogen peroxide after three TBS–0.02% Tween 20 washes (41). To verify the conservation of the epitope recognized by AnaR76A1 among the isolates, acetone-fixed blood smears were stained by direct immunofluorescence as described previously (6) with AnaR76A1, Ana22B1 (positive control), or Tryp1E1 (negative control) as the primary antibody.

Southern blots. *A. marginale* genomic DNA was isolated from infected blood (4), and 1-μg aliquots were digested with one or more restriction enzymes as recommended by the manufacturers. Digested DNA was separated on 1% agarose gels containing 0.1 μg of ethidium bromide per ml and transferred to GeneScreen filters (NEN). The filters were prehybridized in 1 M NaCl–10% dextran sulfate–0.1 mg of sheared herring sperm DNA per ml–1% SDS at 65°C for 4 h. The gene encoding MSP4 has been sequenced (27); to detect only polymorphisms within the *msp4* coding sequence, the DNA probe consisted of a central 580-base segment of *msp4* within the sites recognized by the restriction enzymes. The probe was polymerase chain reaction amplified from genomic DNA, labeled with [α-^{32}P]ATP by use of random hexanucleotide primers and the Klenow fragment of DNA polymerase I (9), and allowed to incubate overnight at 65°C with the prehybridized filters. The filters were washed four times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the last two washes at 65°C and with 1% SDS added, and then washed twice more in 0.1× SSC at room temperature. To ascertain the presence of *msp4* in organisms other than *A. marginale*, dot blots were hybridized as described above, except that 1-μl aliquots of serial dilutions of DNA from different organisms were spotted onto filters which had been rinsed in 2× SSC and air dried. The probe for these dot blots extended from bases 88 to 790 of the *msp4* gene, including all but the last 56 bases of the sequence encoding the mature MSP4 protein. Control blots were probed with a 1.7-kb fragment of the sequence encoding the highly conserved 16S rRNA of *Babesia bigemina* (35) as a positive control for DNA.

MSP4 cloning and expression. The *msp4* gene (27) was amplified from genomic *A. marginale* DNA by use of *Pfu* DNA polymerase (Stratagene). The 5′ primer was constructed from a *Bam*HI restriction site (underlined) immediately followed by the *msp4* sequence encoding the 5′ terminus of mature MSP4 (CCAATGGATCCCCCATGAGTC ACGAAGTGGC) (27); the other primer corresponded to a region 3′ to the estimated end of transcription for *msp4* joined to an *Eco*RI site (underlined) (TAATACGACTCAC TATAGAATTCCCCTGCTCGTGCCTCGTGCTGAAAC). Isolated DNA was digested with *Bam*HI and *Eco*RI and ligated to *Bam*HI-*Eco*RI-double-digested plasmid pGEX-2T (40). The above-described primers were constructed so that the expressed fusion protein, when digested at the thrombin cleavage site, is divided into the entire, mature MSP4 protein, containing only a single extra glycine at the 5′ end, and the glutathione-*S*-transferase protein.

Sure (Stratagene) *Escherichia coli* was transformed with the recombinant plasmid and screened by hybridization with a radiolabeled polymerase chain reaction-amplified *msp4* sequence (bases 211 to 790) as a probe. Several positive colonies were selected by autoradiography; plasmid DNA was isolated and sequenced by use of the Sequenase version 2.0 enzyme (17). One clone (D33) was isolated, grown in Luria-Bertani medium containing 100 μg of ampicillin per ml to an optical density at 600 nm (OD_{600}) of 0.5, and then induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) to form an MSP4–glutathione-*S*-transferase fusion protein. Control, uninduced cells were grown identically, except for the addition of 0.04% glucose to the medium to maintain repression of the promoter. After 4 h, the cells were collected by centrifugation, lysed in protein sample buffer, and subjected to SDS-PAGE and Coomassie blue staining in 10% acetic acid–50% methanol.

Recombinant protein isolation. D33 cells were induced as described above and collected by centrifugation. Since recombinant MSP4 was insoluble, the cells were washed in TEN buffer three times at 19,000 rpm and 4°C; the pellet was solubilized in 0.1 M glycine (pH 9.0)–8 M urea. The pellet was dialyzed against 0.1 M glycine (pH 9.0), and recombinant MSP4 was separated by affinity chromatography with AnaR76A1 as described previously (27). The eluted product was dialyzed against 50 mM $(NH_4)_2CO_3$ (pH 8.0) and quantified by the micro BCA test (Pierce). Digestion of recombinant MSP4 with 3 U of thrombin per ml in $(NH_4)_2CO_3$ for 30 min at room temperature removed the glutathione-*S*-transferase portion. Native MSP4 was isolated from infected erythrocytes by affinity chromatography as described previously (27).

Direct enzyme-linked immunosorbent assays (ELISAs). Isolated native or recombinant MSP4 [100 ng per well in PBS or 150 ng per well in 50 mM $(NH_4)_2CO_3$ (pH 8.0), respectively] was bound overnight onto Immulon 2 microtiter plates at 4°C (14). Other plates were similarly coated with isolated whole initial bodies in PBS (5×10^6 per well). Plates were processed as described previously (14) but with the following modifications: normal or immune bovine sera at 1:40, 1:200, and 1:1,000 dilutions in PBS–1% BSA were used as primary antibodies, and alkaline phosphatase-conjugated goat anti-bovine immunoglobulin G at a 1:3,000 dilution in PBS was used as the secondary antibody. Plates were read at 405 nm after 30 min (recombinant MSP4) or 90 min (native MSP4 and initial bodies).

ELISA test sera. Sera tested by a direct ELISA included five serum samples from cattle immunized with an outer membrane fraction of the Norton (Zimbabwe) isolate of *A. marginale* (42); five serum samples from cattle immunized with an inner membrane fraction of the Norton isolate (42); three serum samples from cattle infected with the Okanogan, Wash., isolate (24); three Food and Agricultural Organization reference serum samples from cattle infected with the Florida, Venezuela, and Argentina isolates (43); eight serum

samples from cattle infected with the Israel *A. marginale* isolate (43); six serum samples from cattle infected with the Florida isolate (43); and six serum samples from cattle immunized with the live *Anaplasma centrale* vaccine for anaplasmosis (43).

Competitive ELISAs. Competitive ELISAs were performed with plates coated with initial bodies as described above (14). No different rabbit sera recognizing native MSP4 were used as test sera, diluted 1:300 in PBS–1% BSA. Normal rabbit serum was used to determine the background, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:20,000 dilution) was used as the secondary antibody. Antisera were preincubated overnight at 4°C with native or recombinant MSP4 in PBS–1% BSA. Percent inhibition was calculated as the OD_{405} {[control (no MSP4 added) − sample]/OD_{405} [control]} × 100.

RESULTS

MSP4 is a surface-exposed parasite protein. MSP4 originally was defined by use of a neutralizing rabbit serum reacting with isolated whole initial bodies (31), which may be contaminated with erythrocyte stroma; thus, it was not known whether MSP4 actually was a parasite protein. Although MSP4 constitutes only a small percentage of the total initial body proteins, immunoprecipitation of ^{35}S-labeled initial bodies with monoclonal antibody AnaR76A1 precipitated a 31-kDa protein, while that with negative control antibody Tryp1E1 did not (Fig. 1A). Since erythrocyte proteins are not metabolically labeled with methionine (3), MSP4 must be of parasite origin.

MSP4 possesses a signal peptide (27); however, whether MSP4 is exposed on the initial body surface is unknown. Radiolabeling of initial body proteins by the lactoperoxidase method may suggest surface exposure of these antigens. Immunoprecipitation of ^{125}I-labeled initial bodies with AnaR76A1 also precipitated a 31-kDa protein (Fig. 1B).

MSP4 is conserved among *A. marginale* isolates. Because *A. marginale* isolates vary widely antigenically (5, 18, 25), conservation of the MSP4 protein was determined by immunoprecipitation and Western blotting (immunoblotting) (Fig. 2). Unlike the MSP2, MSP1a, and MSP1b initial body proteins, which vary widely in size among *A. marginale* isolates (28, 32), the 31-kDa MSP4 protein did not change in apparent molecular size in all the isolates tested. MSP4 also was detected in *A. centrale*, a related rickettsia that infects cattle, but not in the sheep *Anaplasma* species *Anaplasma ovis* (data not shown). Preservation of the MSP4 epitope recognized by monoclonal antibody AnaR76A1 also was established by use of acetone-fixed smears of *A. marginale*-infected blood. Smears of the Florida, South Idaho, Missouri, Oklahoma, and Clarkston, Wash., isolates were found positive by immunofluorescence with the monoclonal antibodies AnaR76A1 and Ana22B1 (positive control) but not with the negative control monoclonal antibody Tryp1E1 (data not shown).

MSP4 is encoded by a single conserved gene. Recently, several *A. marginale* surface proteins were determined to be encoded by members of polymorphic multigene families (4, 28a). Southern blots of *A. marginale* genomic DNA digested with restriction enzymes that cut outside the *msp4* open reading frame (ORF) revealed a single band when *msp4* was used as a probe, suggesting that one copy of the *msp4* gene is present in the *A. marginale* genome (Fig. 3). The two bands in the *Hin*dIII lane resulted from a known *Hin*dIII site within *msp4* (27). The *msp4* gene was conserved among the

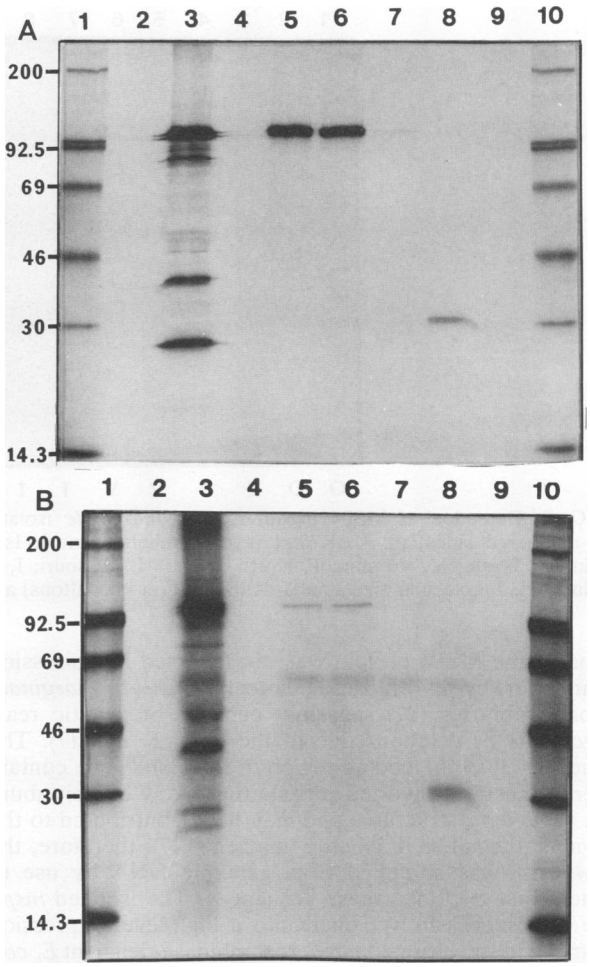

FIG. 1. (A) Immunoprecipitation of [^{35}S]methionine metabolically labeled initial bodies. (B) Immunoprecipitation of ^{125}I surface-labeled initial bodies. Lanes: 1 and 10, ^{14}C-labeled molecular size standards (in kilodaltons); 3, total labeled antigen; 5 and 6, positive control precipitation with Ana22B1 and Ana15D2, respectively; 7, negative control precipitation with antibody Tryp1E1; 8, precipitation with AnaR76A1; 2, 4, and 9, blanks.

five *A. marginale* isolates tested; no restriction length polymorphisms were detected when restriction enzymes that cleave within the *msp4* ORF were used (Fig. 4 and unpublished data). The expected fragment lengths in the Florida isolate were 698 bases (Fig. 4, lane 1) when digestion was done with *Bpm*I and *Sfa*NI or 573 and 263 bases when digestion was done with *Dra*II, *Hin*dIII, and *Afl*III (lane 6).

Southern blots also suggested that the *msp4* gene may be *A. marginale* specific; the gene was detected in *A. marginale* DNA but not in the DNAs of other rickettsiae (*A. ovis*, *Cowdria ruminantium*, *Rickettsia typhi*, and *Haemobartonella felis*), gram-negative bacteria (*E. coli* and *Haemophilus influenzae*), or blood-borne cattle parasites (*Babesia bovis*, *B. bigemina*, and *T. brucei*) (data not shown). The hybridization conditions used were moderately stringent; hybridization experiments with lower-stringency washes might detect more distantly related sequences. Searching the GenBank and EMBL data bases with the *msp4* sequence did not reveal significant similarities with any other DNA sequences or proteins.

Expression and isolation of recombinant MSP4. The gene

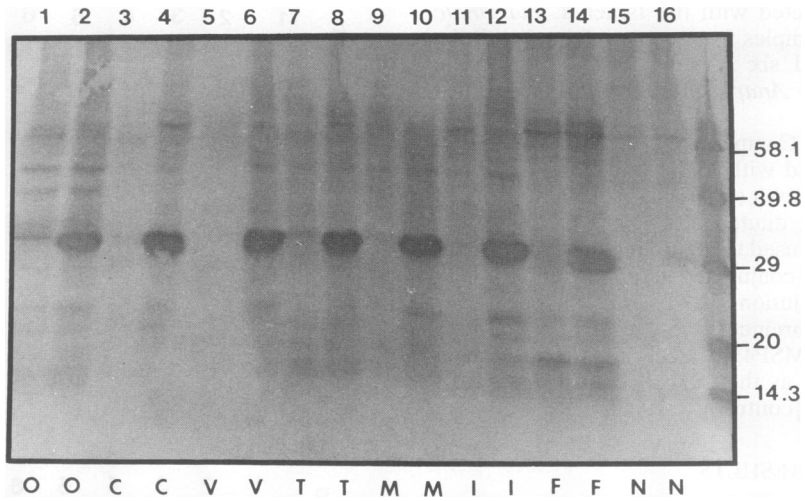

FIG. 2. Detection of MSP4 in different *A. marginale* isolates. Proteins were immunoprecipitated with control antibody Tryp1E1 (odd-numbered lanes) or AnaR76A1 (even-numbered lanes). Isolates or samples are designated as follows: O, Okanogan, Wash.; C, Clarkston, Wash.; V, Virginia; T, North Texas; M, Missouri; I, South Idaho; F, Florida; and N, normal uninfected bovine erythrocytes. Biotin-labeled molecular size standards (Sigma) (in kilodaltons) are on the right.

encoding the MSP4 protein was not detected in expression libraries or by hybridization screening of *A. marginale* genomic libraries (27), possibly because of genetic rearrangements or deletions due to the host, *E. coli* (7). The sequences 3′ to the coding region of the *msp4* gene contain several imperfect inverted repeats that likely exhibit abundant secondary structures and may have contributed to the previous difficulties in cloning this gene (27); therefore, the *msp4* gene was amplified from genomic DNA by use of primers that excluded these sequences. The isolated *msp4* gene was successfully cloned into a high-level expression plasmid and transformed into a recombinase-deficient *E. coli* strain (Sure). The growth rate of the bacteria did not appear to be slowed by the introduction of the *msp4* gene, and induction of the recombinant clone with IPTG resulted in the expression of an insoluble MSP4–glutathione-*S*-transferase fusion protein. Following urea solubilization and renaturation, the isolated protein was recognized by anti-MSP4 monoclonal antibody AnaR76A1 (Fig. 5), demonstrating the conservation of a conformation-sensitive epitope between the native and the recombinant proteins. Furthermore, when digested with thrombin to remove the glutathione-*S*-transferase moiety, the recombinant MSP4 protein comigrated with the native protein in SDS-PAGE (Fig. 5).

Native and recombinant MSP4 share epitopes. Preincubation of rabbit sera recognizing native MSP4 with 250 ng of either recombinant or native MSP4 inhibited 70 to 80% of

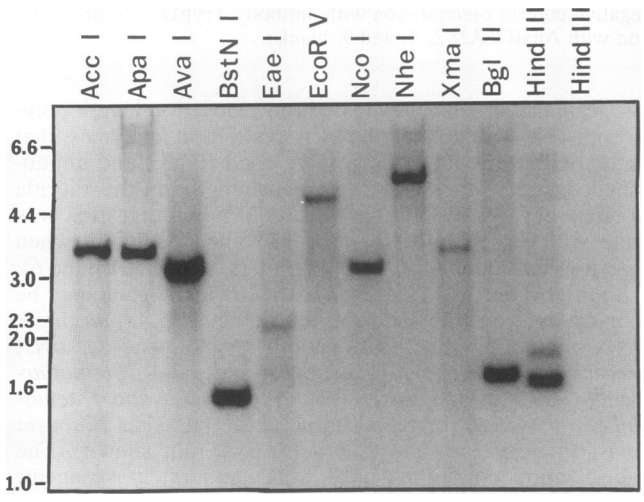

FIG. 3. Southern blot of genomic *A. marginale* DNA digested with restriction enzymes cutting outside the ORF and probed with the *msp4* gene. One microgram of Florida isolate *A. marginale* DNA was digested per well. Bovine DNA digested with *Hin*dIII (rightmost lane) was not recognized by *msp4*. DNA size standards (in kilobases) are on the left.

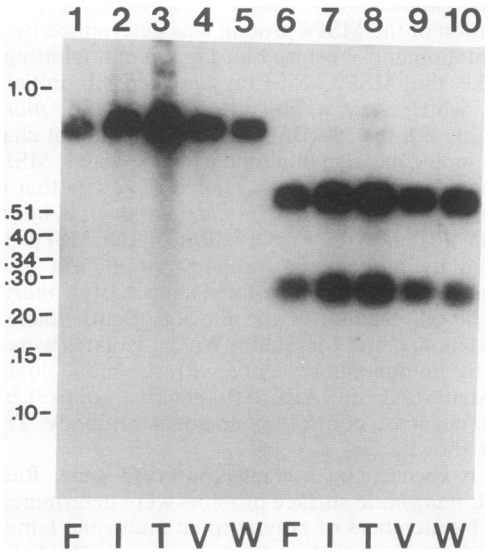

FIG. 4. Southern blot of DNAs from different *A. marginale* isolates digested with restriction enzymes cutting within the ORF and probed with *msp4*. Samples in lanes 1 to 5 were digested with *Bpm*I and *Sfa*NI; samples in lanes 6 to 10 were digested with *Dra*II, *Hin*dIII, and *Afl*III. Isolates are designated as follows: F, Florida; I, South Idaho; T, North Texas; V, Virginia; and W, Okanogan, Wash. DNA size standards (in kilobases) are on the left.

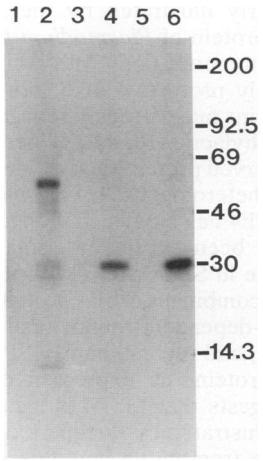

FIG. 5. Immunoprecipitation of labeled native and recombinant MSP4 proteins. ^{125}I-labeled MSP4 protein was precipitated with AnaO58A2 as a negative control (odd-numbered lanes) or AnaR76A1 (even-numbered lanes). Lanes: 1 and 2, recombinant MSP4 fusion protein; 3 and 4, recombinant MSP4 fusion protein after thrombin digestion to remove the glutathione-*S*-transferase portion; 5 and 6, native MSP4 protein isolated from initial bodies. Molecular size standards (in kilodaltons) are on the right.

binding to initial bodies in competitive ELISAs (Fig. 6). Inhibition by recombinant MSP4 was comparable on a molar basis to inhibition by native MSP4. The level of inhibition of control serum (recognizing MSP2) by either native or recombinant MSP4 was less than 3%. This result demonstrates that recombinant and native MSP4 share epitopes as presented on initial bodies.

Immune cattle sera recognize native and recombinant MSP4. Previous work showed that cattle immunized with fractionated *A. marginale* initial body membranes were protected from a virulent challenge (42). Sera from protected cattle immunized with the outer membrane fraction bound strongly to both native and recombinant MSP4 (Table 1), indicating that MSP4 is located in the outer initial body membrane and substantiating a surface location for MSP4. Sera from cattle immunized with the inner membrane fraction did not recognize MSP4.

Postinfection sera varied in their ability to detect native or recombinant MSP4 in ELISAs; only 20 of the 26 cattle serum samples tested had detectable antibody titers to MSP4, and many of these samples recognized MSP4 only at very low titers, suggesting that MSP4 is not immunodominant. Serum samples from five of six *A. centrale*-immunized cattle weakly bound MSP4 in these assays, corroborating the results of Western blotting showing the recognition of a 31-kDa *A. centrale* antigen by anti-MSP4 sera (data not shown).

DISCUSSION

Despite severe economic losses due to anaplasmosis, current vaccine and diagnostic strategies are inadequate. Obstacles include difficulties in the diagnosis of persistently infected carrier animals (23, 46) and the antigenic variability of *A. marginale* isolates (21, 25). Animals which recover from an *A. marginale* infection are protected from a homologous, but not necessarily a heterologous, challenge; the inability of current vaccines to protect against heterologous isolates (20) results in occasional outbreaks of severe clinical disease.

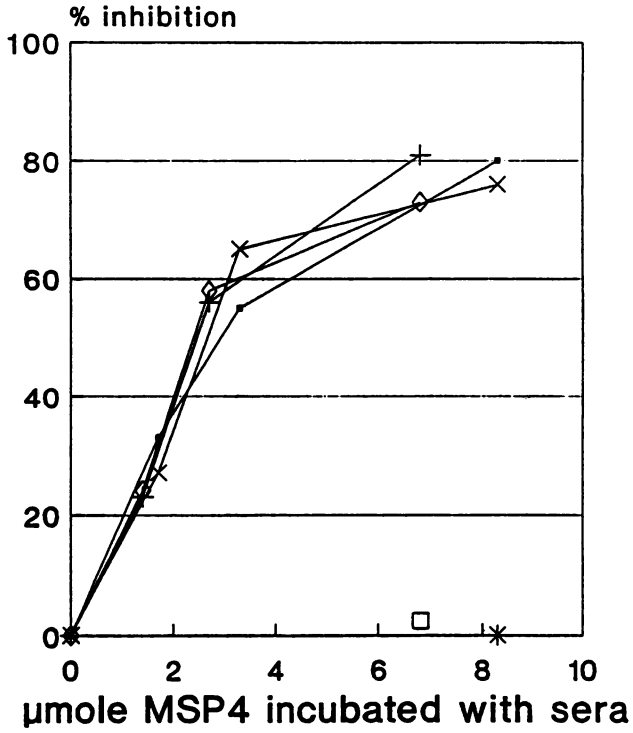

FIG. 6. Inhibition of binding of rabbit anti-MSP4 sera to initial bodies by native or recombinant MSP4 protein. The micromoles of MSP4 protein used for inhibition are listed on the horizontal axis; the percent inhibition of binding is given on the vertical axis. Symbols: × and ■, anti-native MSP4 rabbit sera incubated with native MSP4; ◇ and +, anti-native MSP4 rabbit sera incubated with recombinant MSP4; * and □, anti-MSP2 rabbit serum incubated with native MSP4 or recombinant MSP4, respectively.

Although the mechanism of immunity against anaplasmosis is not known, humoral immunity is probably important, since bovine erythrocytes do not express class I major histocompatibility complex antigens on their surfaces (8). Our strategy for the development of an effective vaccine is the identification of protective epitopes on surface-exposed

TABLE 1. Recognition of *A. marginale* initial bodies, native MSP4, and recombinant MSP4 by bovine immune sera[a]

Serum	Membrane fraction	Titer for:		
		Initial bodies	Native MSP4	Recombinant MSP4
2	Inner	1,000	0	0
6	Inner	1,000	0	0
10	Inner	1,000	0	0
14	Inner	200	0	0
20	Inner	200	0	0
3	Outer	1,000	1,000	1,000
7	Outer	1,000	1,000	1,000
11	Outer	1,000	1,000	1,000
15	Outer	200	1,000	1,000
22	Outer	200	200	1,000

[a] The serum numbers are the inner and outer membrane fraction-immunized cattle described by Tebele et al. (42). The antigens used in the ELISAs are listed across the top (initial bodies or native or recombinant MSP4 protein); values listed under the antigens represent the highest positive titers found in direct ELISAs. Sera were tested at dilutions of 1:40, 1:200, and 1:1,000.

proteins of blood-stage initial bodies. We previously demonstrated that antibodies to *A. marginale* initial bodies can neutralize infectivity (31) and that immunization with isolated surface proteins of *A. marginale* can induce protection (29, 32). Initial work focusing on the MSP2 protein and the MSP1a-MSP1b protein complex demonstrated the ability of these proteins to induce protection against a virulent homologous or one heterologous *A. marginale* challenge (29, 32). The optimal subunit vaccine for anaplasmosis probably will contain several initial body proteins in combination.

Genetic and antigenic conservation of protective proteins may be of critical importance for effective prophylaxis, since *A. marginale* isolates vary widely in their protein structure, antigenicity, morphology, and virulence (3, 16, 19, 21, 25). The MSP2, MSP1a, and MSP1b proteins can vary in apparent size and structure among isolates by as much as 50% on acrylamide gels (28, 32); the antigenic variability of these proteins among isolates may limit their usefulness as potential vaccine candidates. Unlike the MSP1 and MSP2 proteins, MSP4 does not vary among isolates in its apparent molecular mass in SDS-PAGE. Furthermore, the MSP4 protein is apparently conserved among all isolates of *A. marginale* examined, as well as in the related rickettsia *A. centrale*; significantly, *A. centrale* is used effectively as a live vaccine against *A. marginale* in Israel and South Africa (1, 33, 34).

Importantly, MSP4 is conserved not only at the protein level but also at the genetic level. Cyclic parasitemia levels in chronic infections suggest that antigenic variation also may occur within isolates (15). The MSP2 and MSP1b *A. marginale* proteins are encoded by multigene families (4, 28a), which may be involved in intragenic recombination. Differential expression of distinct gene copies further complicates analyses of these proteins. MSP4, on the other hand, is encoded by a single gene, restricting the potential for genetic recombination. Although the exact mechanism(s) of antigenic variation in *A. marginale* is unknown, antigens serving critical functions for parasite survival are unlikely to vary extensively under immune pressure. A conserved protein, such as MSP4, if protective, would appear to be the ideal basis for a subunit vaccine against bovine anaplasmosis.

Previous research demonstrated that immunization of cattle with isolated inner and outer membranes of *A. marginale* initial bodies can each induce protective immunity (42). These membrane fractions differed in their morphology and peptide composition, and different antigenic specificities of antibodies were produced by cattle immunized with either fraction (42). These data established a significant correlation between antibody titers to outer membrane fractions and protective immunity; animals with titers of 10^5 or more against outer membrane proteins were completely protected from microscopically detectable parasitemia. Recognition of MSP4 by sera from outer membrane protein-immunized cattle establishes a membrane location for MSP4 and suggests that MSP4 may be involved in the induction of immunity in these animals. However, the lack of reactivity of sera from inner membrane-protein-immunized cattle suggests that the 31-kDa inner membrane protein may not be MSP4.

Sera from cattle that have been infected with *A. marginale* organisms exhibited variable titers to MSP4. These sera did not consistently recognize either native or recombinant MSP4 in an ELISA or Western blots. Consequently, the native MSP4 antigen may be unrecognized or only weakly immunogenic in some cattle and would not be useful as a diagnostic tool. A high-titer antibody response in infected individuals does not necessarily correlate with protective capability, as clearly illustrated by the immunodominant circumsporozoite protein of *Plasmodium falciparum* (2, 11, 12, 39). Similarly, immunizations with nonimmunodominant proteins were highly protective in *B. bovis* infections (44, 45), while the proteins most strongly recognized by infection sera were not prophylactic (10, 44). Generating an antibody response to a conserved protein such as MSP4 might provide protection against heterologous *A. marginale* isolates.

The gene for MSP4 has now been cloned, and the recombinant protein has been expressed, isolated, and demonstrated to comigrate in SDS-PAGE with the native protein. Both native and recombinant MSP4 proteins are recognized by a conformation-dependent monoclonal antibody and by infected cattle sera, and recombinant MSP4 shares epitopes with the native protein, as expressed on initial bodies. Evidence also suggests that MSP4 is located on the initial body surface, as illustrated by lactoperoxidase labeling and recognition by sera from cattle immunized with outer, but not inner, initial body membranes. Both native and recombinant MSP4 proteins can be recognized by these immune cattle sera. MSP4 is structurally and genetically conserved among *A. marginale* and *A. centrale* isolates and therefore may have greater potential to protect cattle from challenge by heterologous isolates than other *A. marginale* surface proteins, which vary widely in size and structure. We are currently testing the ability of the MSP4 protein to protect cattle from homologous and heterologous *A. marginale* challenges.

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