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Identification and Characterization of a Surface-Exposed, 66-Kilodalton Protein from *Borrelia burgdorferi*

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The surface-exposed antigens of *Borrelia burgdorferi* represent important targets for the development of a protective immune response. We have identified a proteinase K-accessible, 66-kDa protein from *B. burgdorferi* and have demonstrated that at least a portion of this protein is surface exposed. The 66-kDa protein was purified by sequential extraction of spirochetes with butanol and Triton X-114 followed by preparative gel electrophoresis. Polyclonal antibodies developed against the purified 66-kDa protein were *Borrelia* spp. specific, whereas a monoclonal antibody, Route 66, displayed a genospecies-specific pattern of recognition for the 66-kDa protein. N-terminal amino acid sequence was obtained from an internal fragment, a truncated version, and the full-length form of the 66-kDa protein. A search of protein and gene databases for homologous sequences yielded a match with the predicted amino acid sequence from a segment of *B. burgdorferi* chromosomal DNA (P. A. Rosa, D. Hogan, and T. G. Schwan, J. Clin. Microbiol. 29:524–532, 1991). The construction of primers based on this DNA sequence and the N-terminal amino acid sequence allowed the amplification and cloning of the 66-kDa-protein gene. The identity of the cloned gene was verified by the recognition of the expressed gene product by Route 66. Pulsed-field gel electrophoresis and Southern blot analysis were performed to confirm the chromosomal location of the 66-kDa-protein gene. This study describes the identification and cloning of the first chromosomally encoded, surface-exposed protein from *B. burgdorferi*.

Lyme borreliosis is transmitted by ticks belonging primarily to the genus *Ixodes*. The etiologic agent was identified as the spirochete *Borrelia burgdorferi* in 1982 (6). Reforestation and restoration of deer populations have been implicated in the emergence of Lyme borreliosis in recent years (2). Nearly 10,000 cases were reported in 1991, making Lyme borreliosis the most prevalent vector-borne disease in the United States (7). Lyme borreliosis is often initially manifested by the appearance of a characteristic rash, erythema migrans, at the site of tick feeding. Additional early signs of infection are nonspecific and may mimic flu-like symptoms. If untreated, the organisms may disseminate throughout the body, causing widespread organ and tissue pathology, including arthritis, carditis, and neurological dysfunction (40). While Lyme borreliosis is often treatable with antibiotics early in the infection, the report of treatment failures has warranted the development of prophylactic measures, such as vaccines, for the prevention of this disease (25).

The possible risk of autoimmune sequelae and complications associated with whole-cell *B. burgdorferi* vaccines has focused attention on the development of a subunit vaccine (16, 37). Considerable effort is under way to identify the antigens important in eliciting a protective immune response to *B. burgdorferi* (8). Humoral immunity to surface-exposed antigens appears to be an important component of the development of a protective immune response (11). Antibodies to outer surface protein A (OspA) or OspB are directly cytolytic for *B. burgdorferi* (34). Immunization with OspA, OspB, OspC, or OspF has proven effective in protecting animal models challenged with *B. burgdorferi* (10, 21, 26). Unfortunately, the genes encoding these antigens are located on plasmids and display a much higher degree of sequence heterogeneity than do chromosomally encoded genes (43). Moreover, the expression level of these antigens may be reduced or nonexistent because of transcriptional regulation or shedding of the plasmid (19, 23). Therefore, the identification of a chromosomally encoded, surface-exposed antigen that provides broad cross-protection would be highly desirable as a constituent of a subunit vaccine.

We have attempted to identify novel surface-exposed antigens from *B. burgdorferi*. Using surface proteolysis as a preliminary screening assay, we identified a 66-kDa antigen that appears to be partially exposed on the outer surface of *B. burgdorferi*. Immunological and biochemical analyses indicated that this antigen was highly conserved and that the gene encoding this antigen was chromosomal in origin. Limited amino acid sequence analysis enabled the identification and cloning of the gene encoding the 66-kDa antigen.

MATERIALS AND METHODS

Organisms. The genospecies designations and origins of the *Borrelia* isolates used in this study are listed in Table 1. All *Borrelia* isolates were maintained in Barbour-Stoenner-Kelly medium (BSKII) and subcultured every 3 to 4 days. Isolates were kindly provided by John F. Anderson (Connecticut Agricultural Experiment Station), Paul Duffy (California Department of Health Services), Russell C. Johnson (University of Minnesota), Robert S. Lane (University of California, Berkeley), Tom G. Schwan (Rocky Mountain National Laboratory), and Richard L. Walker (California Veterinary Diagnostic Laboratory). *Leptospira interrogans* serovar pomona was grown in 10% EMJH leptospiral enrichment medium (Difco Laboratories, Detroit, Mich.). *Treponema denticola* (ATCC 33520) and *Serpulina hyodesentariae* (ATCC 27164) were provided by Richard L. Walker (California Veterinary Diagnostic Laboratory) as actively growing cultures and were used immediately to prepare lysates for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (see below).

Proteinase K digestion. Proteinase K digestion was performed as described by Barbour et al. (4). Actively growing SON 188 spirochetes were washed twice and resuspended in phosphate-buffered saline supplemented with 5 mM $MgCl_2$ (PBS-Mg). Proteinase K dissolved in PBS-Mg was added to a final concentration of 0.4 mg/ml, and the spirochetes were incubated for 30 min at room temperature. The proteinase K was then inactivated by addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mg/ml.

Purification of full-length and truncated 66-kDa antigens. Purification of the

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TABLE 1. *Borrelia* isolates used in this study

Borrelia sp.	Isolate	Genospecies[a]	Host (geographic origin)
B. burgdorferi	SON 188	I	*Ixodes pacificus* (California)
	B31	I	*I. scapularis* (New York)
	297	I	Human CSF[b] (Connecticut)
	SH-2-82	I	*I. scapularis* (New York)
	CA-2-87	I	*I. pacificus* (California)
	IP-2	I	Human CSF (France)
	P/Bi	II	Human CSF (Germany)
	K48	II	*I. ricinus* (Czechoslovakia)
	G25	II	*I. ricinus* (Sweden)
	20047	II	*I. ricinus* (France)
	BO23	III	Human skin (Germany)
	VS461	III	*I. ricinus* (Switzerland)
	PGau	III	Human skin (Germany)
	IP3	III	*I. persulcatus* (Russia)
	25015	?	*I. scapularis* (New York)
	DN 1-2-7	?	*I. pacificus* (California)
B. anserina	Esparto	NA[c]	Chicken (California)
B. coriaceae	Co53	NA	*Ornithodoros coriaceae* (California)
B. hermsii	ATCC 3509	NA	*O. hermsi* (California)

[a] I, *B. burgdorferi* sensu stricto; II, *B. garinii*; III, *B. afzelii*.
[b] CSF, cerebral spinal fluid.
[c] NA, not applicable.

66-kDa antigen involved a three-step procedure including (i) butanol extraction, (ii) Triton X-114 extraction, and (iii) gel purification. Purification of the truncated form of the 66-kDa antigen was preceded by proteinase K digestion as described above.

A 250-ml culture of actively growing SON 188 spirochetes was washed twice in PBS-Mg, and the pellet was resuspended in 800 μl of PBS-Mg. Two volumes of butanol was added, and the spirochetes were vortexed intermittently over 30 min. Following centrifugation at 16,000 × *g* for 20 min, the aqueous and organic phases were discarded and the insoluble interface was retained. This material was washed twice in Tris-buffered saline (TBS) and resuspended in TBS–2% Triton X-114. This extraction was performed at 4°C for 2 h with constant agitation. Following extraction, the insoluble material was removed by centrifugation at 16,000 × *g* for 20 min at 4°C. The supernatant was warmed to 37°C to allow phase separation and then centrifuged at room temperature, and the aqueous layer containing the partially purified 66-kDa antigen was retained for gel purification.

The amount of protein present in the Triton X-114 aqueous phase was quantitated by the bicinchoninic acid assay (Pierce, Rockford, Ill.), diluted to a concentration of 250 μg/ml in SDS-PAGE sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol [pH 6.8]), and subjected to preparative SDS-PAGE on 10% acrylamide gels. Electrophoresis was performed for 4 h at 35 mA per gel, and the protein bands were identified by soaking the gels in 0.25 M KCl for 10 min. The full-length and truncated forms of the 66-kDa protein were excised, and the proteins were eluted in an electrophoretic concentrator (LKB, Bromma, Sweden). The eluted proteins were concentrated in a Centricon 10 (Amicon, Beverly, Mass.) and diafiltrated into TBS, and the protein contents were quantitated by the bicinchoninic acid assay.

Protein sequence analysis. Full-length and truncated forms of the 66-kDa antigen were purified by butanol and Triton X-114 extraction for N-terminal sequence analysis. The Triton X-114 aqueous-phase proteins were resuspended in SDS-PAGE sample buffer, heated to 65°C for 15 min, and separated on a 10% minigel for 60 min at 150 V. The proteins were electroblotted for 60 min at 35 mA onto a Problot membrane (Applied Biosystems, Foster City, Calif.), using a semidry blotting apparatus (LKB). Transferred proteins were detected by immersing the membrane in 0.25% Coomassie blue in 50% methanol for 45 s and destaining in several washes of 50% methanol–10% acetic acid. The bands representing the full-length and truncated forms of the 66-kDa protein were excised and submitted to the Protein Structure Laboratory (University of California, Davis) and sequenced with an Applied Biosystems 470 amino acid sequencer.

Internal amino acid sequence was obtained by partial digestion of the purified full-length and truncated forms of the 66-kDa protein with endoproteinase Lys-C (Promega, Madison, Wis.). Twenty micrograms of each gel-purified protein was incubated for 1 h at 37°C with 0.2 μg of enzyme. The reaction was stopped by the addition of SDS-PAGE sample buffer and by boiling the sample for 5 min. Protein fragments were resolved on a 12% acrylamide gel and processed for amino acid sequence analysis as described above.

Antibodies. For antiserum production, six C3H/HeN mice (Simonsens Laboratory, Gilroy, Calif.) were immunized with the gel-purified, full-length 66-kDa antigen. Each mouse was primed with a 200-μl intraperitoneal inoculation containing 5 μg of antigen emulsified in complete Freund's adjuvant. The mice were subsequently boosted with the same quantity of immunogen in incomplete Freund's adjuvant on days 14, 35, and 56 following the initial inoculation. The mice were bled, and serum was collected 7 days after the final boost.

For monoclonal antibody development, BALB/c mice (Simonsens Laboratory) were immunized as described above. However, only a single boost was administered. Five days following this boost, a mouse was sacrificed and the spleen was removed aseptically for hybridoma production. Cell fusion was performed as described by Galfre et al. (12). Briefly, 10^7 splenocytes were fused with 10^6 P3X63-Ag8.653 myeloma cells in the presence of 50% polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, Ind.). Fused cells were resuspended in Dulbecco's modified Eagle medium containing 20% fetal bovine serum, and hybridomas were selected by the presence of 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Boehringer Mannheim). Fourteen days after cell fusion, supernatants were screened for the presence of antibodies specific for the 66-kDa antigen by Western blot (immunoblot) analysis (see below). Hybridomas that tested positive by Western blotting were expanded and cloned twice by limiting dilution. Isotype was determined by using an Isostrip kit (Boehringer Mannheim) according to the manufacturer's instructions.

A monoclonal antibody recognizing flagella (H9724) was kindly provided by Richard L. Walker (California Veterinary Diagnostic Laboratory). The preparation of mouse antisera to recombinant OspA, OspB, OspC, OspD, and P83 was previously reported (27). Mouse antisera specific for recombinant IpLA-7 and P39 were similarly derived (28, 29).

Affinity purification of anti-66-kDa protein serum. Affinity purification of antiserum was performed by the method of Smith and Fisher (39). One hundred micrograms of *B. burgdorferi* 25015 lysate was loaded on a preparative 10% acrylamide minigel, and the proteins were resolved by electrophoresis at 150 V for 1 h. The proteins were transferred onto a nitrocellulose membrane for 1 h at 35 mA, using a semidry blotting apparatus (LKB). The nitrocellulose membrane was immersed in BLOTTO (50 mM Tris, 150 mM sodium chloride, 0.05% Tween 20, 5% nonfat milk [pH 7.5]) for 1 h to block nonspecific reactivity and then probed for 16 h with a 1:1,000 dilution of antiserum diluted in BLOTTO. The membrane was washed in TBS with 0.05% Tween 20 (TTBS) and incubated for 1 h with a 1:5,000 dilution of alkaline phosphatase-labeled protein A (Sigma, St. Louis, Mo.). The membrane was washed in TTBS and developed by the addition of bromochloroindolyl phosphate (BCIP)-nitroblue tetrazolium for 10 min. The band representing the 66-kDa antigen was excised, and the antibodies were eluted by immersing the nitrocellulose strip in 100 mM glycine (pH 2.5). The nitrocellulose was removed, and the pH of the solution was neutralized by the addition of 1.5 M Tris (pH 8.8). The antibody solution was immediately diluted 1:10 in BLOTTO and used as a probe.

Western blot analysis. All spirochetes were washed twice and resuspended in PBS-Mg. Protein content was measured by the bicinchoninic acid assay, and the spirochetes were lysed in SDS-PAGE sample buffer. Three micrograms of each lysate was separated on a 10 or 12% acrylamide gel and transferred to a nitrocellulose membrane as described above. The nitrocellulose membranes were blocked in BLOTTO and incubated for 16 h with the affinity-purified antibodies, a 1:5,000 dilution of mouse antisera, or a 1:50 dilution of a monoclonal antibody. Antibody binding was detected with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and BCIP-nitroblue tetrazolium.

Cloning of the gene encoding the 66-kDa protein. The gene encoding the 66-kDa protein was amplified by PCR and cloned into the pMal c2 vector (New England Biolabs, Beverly, Mass.) as previously described (27). The primers used for amplification were primer 1 (5′-GCTGATGCTCTTAAAGAAAAAGATAT ATTTAAAACA) and primer 2 (5′-CGCTGCAGTTAAGCTTCCGCTGTAG GCTATTTTG). Total DNA from isolate SON 188 served as template for the PCR. Amplification was performed for 30 cycles, using the following parameters: 1 min of denaturation at 94°C, 2 min of annealing at 45°C, and 3 min of extension at 72°C. Following amplification, the size of the PCR product was confirmed by agarose gel electrophoresis. Cloning of the PCR product was facilitated by the incorporation of a *Pst*I site into primer 2 (underlined above). The PCR product was treated with the Klenow fragment of DNA polymerase, excised with *Pst*I, and phosphorylated with T4 polynucleotide kinase. The vector, pMal c2, was prepared for ligation by cutting with *Xmn*I and *Pst*I and dephosphorylating with calf intestinal alkaline phosphatase. The PCR product and vector were ligated for 24 h at 16°C with T4 DNA ligase. Following ligation, the construct was transformed into competent *Escherichia coli* DH5α (Gibco/BRL, Gaithersburg, Md.), and a clone expressing the 66-kDa-protein gene product was detected by Western blot analysis.

Pulsed-field gel electrophoresis and Southern blot hybridization. Pulsed-field gel electrophoresis was performed as previously described (44). Spirochetes were embedded in 0.5% low-melting-temperature agarose plugs at a concentration of 8×10^8 organisms per ml. The plugs were incubated in lysis buffer (50 mM Tris [pH 8], 50 mM EDTA, 1% SDS) containing 1 mg of proteinase K per ml for 48 h at 50°C. Insert slices containing DNA derived from approximately 4×10^7 spirochetes were added to a 1% agarose gel, and electrophoresis was performed with an LKB 2015 Pulsaphor system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The gel was run at 330 V for 24 h with a switch time of 5 s. Electrophoresed DNA was transferred to a nylon membrane (Hybond N^+; Amersham,

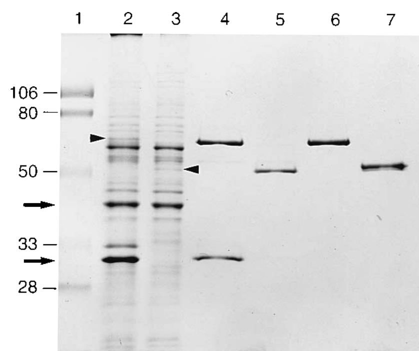

FIG. 1. Purification and identification of the full-length and truncated forms of the 66-kDa protein. Proteins were resolved on a 10% acrylamide gel and stained with Coomassie blue. Lane 1, molecular mass standards, positions of which are indicated in kilodaltons; lane 2, total lysate of SON 188; lane 3, total lysate of proteinase K-treated SON 188; lane 4, Triton X-114 aqueous-phase proteins derived from untreated SON 188; lane 5, Triton X-114 aqueous-phase proteins derived from proteinase K-treated SON 188; lane 6, gel-purified 66-kDa protein derived from untreated SON 188; lane 7, gel-purified, truncated form of the 66-kDa protein derived from proteinase K-treated SON 188. Arrowheads illustrate the positions of the full-length (lane 2) and truncated (lane 3) forms of the 66-kDa protein; arrows indicate the locations of the flagellum protein (upper arrow) and OspA (lower arrow).

Arlington Heights, Ill.), using the Vacugene 2016 system (LKB Pharmacia, Piscataway, N.J.). Digoxigenin-labeled DNA probes to the *P83*, *ospA*, and 66-kDa-protein genes were prepared by PCR (9). Primer sets for the *P83* and *ospA* genes were previously reported (27). Primers for the 66-kDa-protein gene and amplification conditions were described above. Nucleic acid hybridization was performed at 45°C for 16 h, and the hybridized probes were detected by chemiluminescence using the Genius system (Boehringer Mannheim).

RESULTS

Proteinase K digestion and purification of the 66-kDa protein. Figure 1 demonstrates the susceptibility of OspA (31 kDa) and OspB (33 kDa) to proteinase K digestion, whereas the periplasmic flagellar protein (41 kDa) appeared relatively unaffected. An additional proteinase K-susceptible target was observed for a protein of 66 kDa. The disappearance of the 66-kDa protein band and the appearance of a new band of 50 kDa upon proteinase K treatment suggested that the two proteins may be related.

The 66- and 50-kDa proteins were highly enriched by a two-step purification involving a modification of the butanol extraction technique of Gondolf et al. (13) followed by Triton X-114 extraction. This combination of extraction procedures yielded two major proteins of 66 and 31 kDa (OspA) when applied to untreated spirochetes (Fig. 1, lane 4) and a single major band of 50 kDa when extraction was preceded by proteinase K treatment (Fig. 1, lane 5). The addition of a gel purification step further enriched the 66- and 50-kDa proteins and provided material that was approximately 95% pure as determined by SDS-PAGE (Fig. 1, lanes 6 and 7).

Protein sequencing. N-terminal analysis of the 66- and 50-kDa proteins yielded identical amino acid sequences (Table 2). This information indicated that the 50-kDa protein was a truncated version of the 66-kDa protein and that the proteinase K-susceptible moiety represented the carboxy-terminal end of the 66-kDa antigen. Homology searches by analysis of European Molecular Biology Laboratory (EMBL) and National Biomedical Research Foundation (NBRF) databases yielded no significant matches. However, a review of literature provided a 72% homology match with the N-terminal sequence of a *B. burgdorferi* protein of 60 kDa reported by Mensi et al. (20) (Table 2).

TABLE 2. Comparison of amino acid sequences

Protein	Amino acid sequence	Reference
66-kDa N terminus	ADALKEKDIFKTNPWMPTFGFE	This study
50-kDa N terminus[a]	ADALKEKDIFKTNPWMPTFGFE	This study
60-kDa N terminus	ADALGEKDIFKTNPLYGG[b]	20
66-kDa internal[c]	AE_IFDPNGNALNF[d]	This study
PCR target		
B31	AE_IFD[d]	32
G2	AEDIFDPNGNALNF	32

[a] The truncated form of the 66-kDa protein obtained from spirochetes treated with proteinase K.
[b] Two choices (K and G) were reported at amino acid position 11.
[c] The N-terminal sequence of a 19-kDa internal fragment of the 66-kDa protein generated by partial digestion with endoproteinase Lys-C.
[d] Space added for optimal sequence alignment.

Partial proteolytic digestion of the full-length and truncated forms of the 66-kDa protein with endoproteinase Lys-C yielded similar patterns of fragmentation (Fig. 2). However, two bands were unique to the pattern of proteolysis observed for the full-length form of the 66-kDa protein. Since the truncated and full-length forms of the 66-kDa protein were identical at the N terminus, these bands presumably represented the carboxy end of the molecule, and one of these fragments (19 kDa) was selected for sequence analysis. Thirteen amino acid residues were obtained from the N terminus of this fragment and are shown in Table 2. A search of the EMBL and NBRF databases for homologous sequences yielded a significant match with the predicted amino acid sequence of a *B. burgdorferi* PCR target described by Rosa et al. (32). The amino acid sequence was identical at 5 of 5 amino acid residues predicted from the nucleotide sequence of *B. burgdorferi* isolate B31 and 13 of 13 amino acids (omitting a single amino acid from the G2 sequence) derived from the nucleotide sequence of *B. burgdorferi* isolate G2 (Table 2).

Antibody specificities. Serum obtained from mice immunized with the 66-kDa protein possessed an antibody repertoire that recognized a 66-kDa band and a 31-kDa band (OspA) upon Western blot analysis of SON 188 lysate (data not shown). The recognition of OspA by this serum probably reflected the presence of undetected amounts of OspA contaminating the 66-kDa immunogen. To eliminate antibodies recognizing OspA and to enrich for antibodies specific for the

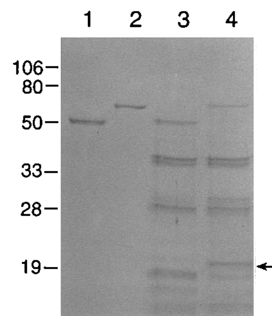

FIG. 2. Endoproteinase Lys-C digest of the truncated and full-length forms of the 66-kDa protein. Proteins were resolved on a 12% acrylamide gel, transferred to a Problot membrane, and stained with Coomassie blue. Lane 1, truncated form of the 66-kDa protein; lane 2, full-length 66-kDa protein; lane 3, Lys-C digest of the truncated form of the 66-kDa protein; lane 4, Lys-C digest of the full-length 66-kDa protein. The arrow indicates the location of the 19-kDa fragment submitted for N-terminal amino acid sequence analysis. Molecular masses are provided in kilodaltons.

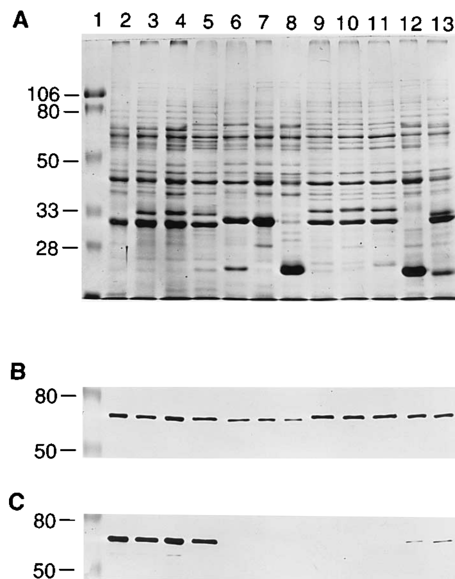

FIG. 3. SDS-PAGE and Western blot analysis of *B. burgdorferi* isolates probed with anti-66-kDa antibodies. Three micrograms of each *B. burgdorferi* lysate was resolved on a 10% acrylamide gel and either stained with Coomassie blue (A) or transferred to nitrocellulose (B and C). Western blot analyses were performed with affinity-purified anti-66-kDa protein antibodies (B) and monoclonal antibody Route 66 (C) as probes. Lane 1 contains prestained molecular mass markers, positions of which are indicated in kilodaltons. Lysates were loaded to lanes 2 to 13 in the following order: 297, SH-2-82, CA-2-87, and IP-2 (genospecies I), K48, G25, and 20047 (genospecies II), VS461, PGau, and IP3 (genospecies III), and 25015 and DN 1-2-7 (atypical isolates).

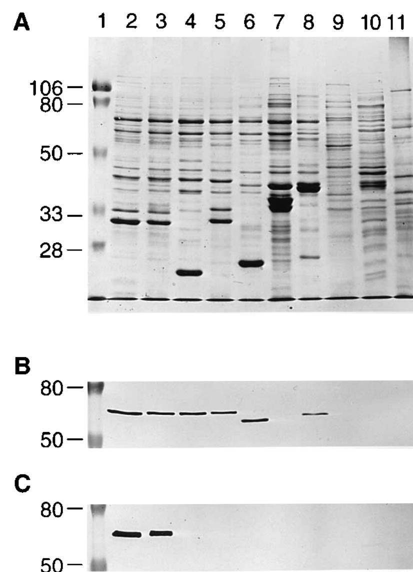

FIG. 4. Detection of the 66-kDa antigen among spirochetes. Three micrograms of each spirochetal lysate was resolved on a 10% acrylamide gel and either stained with Coomassie blue (A) or transferred to nitrocellulose (B and C). Western blot analyses were performed with affinity-purified anti-66-kDa protein antibodies (B) and monoclonal antibody Route 66 (C) as probes. Lane 1 contains prestained molecular mass markers, positions of which are indicated in kilodaltons. Lysates were loaded to lanes 2 to 11 in the following order: *B. burgdorferi* isolates SON 188 and B31 (genospecies I), P/Bi (genospecies II), and BO23 (genospecies III), *B. anserina*, *B. coriaceae*, *B. hermsii*, *T. denticola*, *S. hyodysenteriae*, and *L. interrogans* serovar pomona.

66-kDa antigen, the serum was affinity purified by using strain 25015, a *B. burgdorferi* isolate lacking expression of OspA (28). Antibodies purified in this manner recognized only a single band upon Western blot analysis (Fig. 3 and 4). Screening of a panel of *B. burgdorferi* isolates for the presence of the 66-kDa antigen revealed that all isolates tested, including representatives of all three genospecies, expressed an immunoreactive 66-kDa band (Fig. 3B). The extent to which the 66-kDa antigen was conserved among spirochetes was tested by probing a panel representing four genera of spirochetes (Fig. 4B). An immunoreactive band ranging in size from 62 to 66 kDa was observed among all borreliae tested, whereas no cross-reactivity was observed with spirochetes of other genera. The 66-kDa band associated with *B. coriaceae* was very faint, yet detectable, relative to the specific immunostaining observed with the other *Borrelia* isolates. Thus, the affinity-purified anti-66-kDa antigen antibodies appeared to be genus specific.

The development of monoclonal antibodies further facilitated the characterization of the 66-kDa antigen. Seven hybridomas secreting antibodies specific for the 66-kDa protein were identified upon initial screening by Western blot analysis. All seven hybridomas secreted antibodies recognizing both truncated and full-length versions of the 66-kDa antigen, indicating that these antibodies recognized an epitope located on the N-terminal portion of the 66-kDa antigen (data not shown). One of these hybridomas, Route 66, was selected for further characterization and established to be of the immunoglobulin M isotype. The specificity of Route 66 was determined by using a panel of spirochetes identical to those described above for the affinity-purified antibodies. Route 66 produced specific immunostaining with isolates belonging to genospecies I (*B. burgdorferi* sensu stricto) and not to isolates representing either genospecies II (*B. garinii*) or genospecies III (*B. afzelii*) (Fig. 3C). Recognition of other *Borrelia* spp. or other non-*Borrelia* spirochetes was not observed (Fig. 4C). However, Route 66 demonstrated reduced immunostaining of two atypical isolates, 25015 and DN 1-2-7, suggesting that these North American isolates are more closely related to genospecies I isolates than to isolates representing genospecies II or III (Fig. 3C). Similar findings at the genetic level were recently reported by Assous et al. (1).

Proteinase K accessibility and surface localization. To establish the specificity of proteinase K accessibility as a measure of surface exposure, we examined by Western blot analysis the fate of a panel of known surface-exposed proteins and internal proteins following the exposure of intact *B. burgdorferi* to proteinase K. The surface-exposed proteins consisted of OspA, OspB, OspC, and OspD (4, 5, 22, 24), whereas the internal proteins included IpLA-7, P39, P83, and flagella (18, 41, 42). Figure 5 illustrates the significant reduction in immunostaining observed for OspA, OspB, OspC, and OspD following treatment of intact spirochetes with proteinase K. In contrast, little or no change in band intensity was observed for any of the internal proteins after surface proteolysis with proteinase K. These data validate the specificity of proteinase K accessibility as a technique for determining surface exposure. As described earlier and illustrated again in Fig. 5, the 66-kDa protein undergoes a reduction in molecular mass upon exposure of the spirochetes to proteinase K. Given the relationship between proteinase K accessibility and surface localization, we conclude that at least a portion of the 66-kDa protein was surface exposed.

To demonstrate that the internal proteins and the truncated form of the 66-kDa protein are not inherently resistant to proteinase K, we conducted proteinase K accessibility experiments similar to those described above but with the addition of a nonionic detergent, Triton X-100. Following disruption of

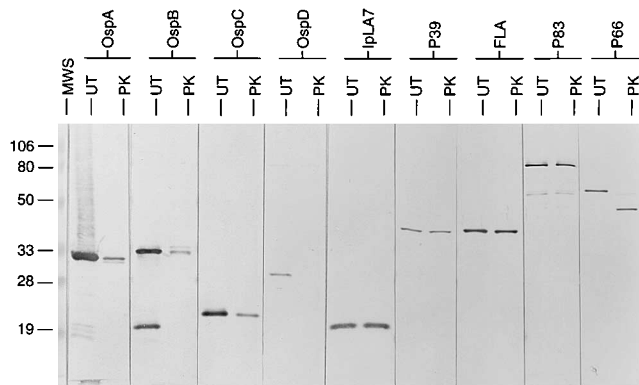

FIG. 5. Surface localization and proteinase K accessibility. Proteins from untreated (UT) and proteinase K-treated (PK) SON 188 were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antisera or monoclonal antibodies. A panel of antibodies recognizing surface-exposed proteins (OspA, OspB, OspC, and OspD) or internal proteins (IpLA-7, P39, P83, and flagella [FLA]) were used to demonstrate the specificity of proteinase K accessibility in defining surface localization. Proteinase K accessibility was illustrated by a reduction in band intensity found among treated spirochetes relative to band intensity observed for untreated spirochetes. The reduction in molecular mass of the 66-kDa protein following the treatment of SON 188 with proteinase K indicated that at least a portion of this protein was surface exposed. MWS, molecular weight standards, positions of which are indicated in kilodaltons.

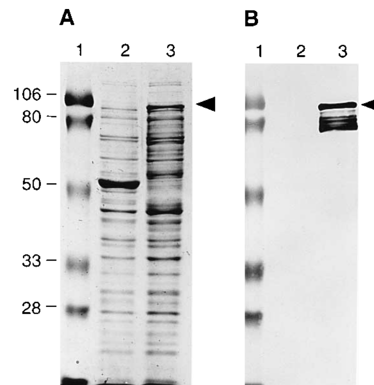

FIG. 6. Expression and immunoreactivity of the cloned 66-kDa-protein gene product. Proteins were resolved on a 10% acrylamide gel and stained with Coomassie blue (A) or transferred to nitrocellulose and probed with monoclonal antibody Route 66 (B). Lane 1, prestained molecular mass markers, positions of which are indicated in kilodaltons; lane 2, lysate of *E. coli* transformed with vector only; lane 3, lysate of *E. coli* clone transformed with the 66-kDa-protein gene. Arrowheads indicate locations of the 66-kDa fusion protein.

the spirochetal outer membrane by Triton X-100 and exposure to proteinase K, none of the internal proteins or the truncated form of the 66-kDa protein could be detected by Western blot analysis (data not shown).

Cloning and expression of the 66-kDa gene. The homology match obtained for the internal fragment of the 66-kDa protein suggested that a portion of the chromosomal DNA sequence described by Rosa et al. (32) likely encoded the C-terminal end of the 66-kDa protein. We used this information to design a primer (primer 2) incorporating the termination codon and delimiting the 3′ end of the open reading frame reported by Rosa et al. (32). Because their sequence lacked the 5′ end of the open reading frame, we synthesized a primer (primer 1) based on the N-terminal amino acid sequence obtained for the 66-kDa protein. The design of this primer accounted for the preferred codon usage of *B. burgdorferi* (14). An amplification reaction using these primers and total DNA from SON 188 resulted in a PCR product of the size predicted to encode a 66-kDa protein (approximately 1,800 bp; data not shown).

Cloning of the PCR product into the pMal c2 vector was facilitated by the incorporation of a unique *Pst*I site into primer 2. The PCR product was inserted downstream from the *malE* gene, resulting in the expression of a fusion protein consisting of maltose-binding protein (encoded by the *malE* gene) followed by the 66-kDa protein (carboxy-terminal end of the fusion protein). A clone expressing an immunoreactive fusion protein of the appropriate size (100 kDa) was identified by SDS-PAGE and Western blotting (Fig. 6). The recognition of this fusion protein by monoclonal antibody Route 66 verified the identity of the cloned gene as that encoding the 66-kDa protein.

Chromosomal localization of the 66-kDa-protein gene. The localization of the gene encoding the 66-kDa protein to the chromosome of *B. burgdorferi* was confirmed by pulsed-field gel electrophoresis and Southern blot hybridization (Fig. 7). Probes specific for the *P83* and *ospA* genes were used to identify chromosomal and plasmid DNAs, respectively (3, 15). A probe representing the cloned 66-kDa-protein gene hybridized with the chromosomal DNA.

DISCUSSION

We have identified a unique surface-exposed protein from *B. burgdorferi*. Surface localization of at least a portion of the 66-kDa protein was established by proteinase K accessibility experiments. We have validated the use of proteinase K accessibility for surface localization by testing a panel of well-characterized *B. burgdorferi* proteins, many of which have been investigated by immunoelectron microscopy (4, 5, 18, 24, 41). Other investigators have reported the putative surface localization of a 66-kDa protein from *B. burgdorferi*. Barbour et al. (4) observed that proteinase K treatment of intact spirochetes resulted in the loss of the 31-kDa (OspA), 34-kDa (OspB), and 66-kDa proteins. Luft et al. (17) performed surface labeling experiments using ^{125}I and biotin to identify the surface-ex-

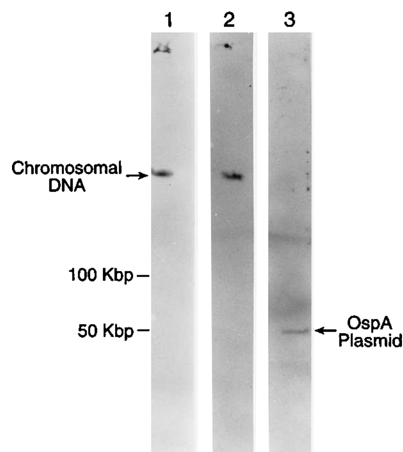

FIG. 7. Chromosomal localization of the 66-kDa-protein gene. *B. burgdorferi* DNA was resolved by pulsed-field gel electrophoresis and transferred for Southern blot analysis. Digoxigenin-labeled gene probes were amplified by PCR, and hybridization of the probes to the transferred DNA was detected by chemiluminescence. Lane 1, *P83* gene probe; lane 2, 66-kDa-protein gene probe; lane 3, *ospA* gene probe. Positions of molecular weight markers are shown at the left.

posed proteins of *B. burgdorferi*, one of which migrated at 66 kDa and displayed an acidic pI.

Amino acid sequence comparison of the 66-kDa protein with a 60-kDa *B. burgdorferi* protein described by Mensi et al. (20) indicated that the two proteins were closely related at the N terminus. Serological analysis by this group demonstrated that this 60-kDa protein was frequently recognized by sera from patients with Lyme disease. Similarly, Sadziene et al. (34) observed by Western blot analysis that 12 of 15 patients with Lyme disease recognized a protease-susceptible 66-kDa antigen. Most notably, these investigators found that sera exhibiting high levels of growth inhibiting activity for *B. burgdorferi* also possessed antibodies to the 66-kDa antigen in a higher frequency of cases than antibodies to all other antigens studied except flagella. Scriba et al. (36) have described human monoclonal antibodies with dual specificity for P39 and a 66-kDa protein that also exhibit bactericidal activity. In a previous study, we were unable to attribute any borreliacidal activity to an antiserum generated to a recombinant P39 antigen (29). This observation may indicate that the lytic activity of the human monoclonal antibodies developed by Scriba et al. (36) is directed toward the 66-kDa antigen and not P39. We are currently developing antisera to the recombinant 66-kDa protein to directly address whether this antigen is a target for borreliacidal and protective antibodies.

Internal sequence information derived from a Lys-C fragment of the 66-kDa protein was identical to a segment of predicted amino acid sequence from a PCR target described by Rosa et al. (32). These investigators randomly cloned and sequenced a fragment of chromosomal DNA from *B. burgdorferi* for use in the development of a diagnostic assay for Lyme disease (33). This DNA sequence included an open reading frame encoding 434 amino acids presumably from the 3′ end of a structural gene. Sequencing of this region from four *B. burgdorferi* isolates representing very different biological and geographical origins resulted in greater than 90% nucleotide homology. Sequence analysis of a homologous region from *B. hermsii* was found to have 70% nucleotide homology with the *B. burgdorferi* sequence. PCR analysis using specific primer sets derived from within this stretch of sequenced DNA allowed these investigators to design (i) a *B. hermsii*-specific assay, (ii) a *B. burgdorferi*-specific assay, and (iii) a *B. burgdorferi* type-specific assay. Not surprisingly, we have observed similar results at the antigenic level. Specificity studies using affinity-purified antibodies indicated that the 66-kDa protein was conserved among *Borrelia* spp. but not present among other spirochete groups. All *B. burgdorferi* isolates tested in our study possessed an immunoreactive 66-kDa antigen when probed with these polyclonal antibodies. Whereas the polyclonal antibodies identified a *Borrelia*-specific antigen, monoclonal antibody Route 66 recognized a genospecies-specific epitope. Taken together, these data may prove useful in the design of diagnostic tools that discriminate at both the genus and species levels.

With the N-terminal amino acid sequence of the 66-kDa protein determined and the availability of DNA sequence presumably encoding the C-terminal portion of the protein, we were able to clone the entire coding region of the 66-kDa gene by using PCR technology. The identity of the cloned PCR product was confirmed by recognition of the expressed fusion protein by Route 66. We are currently sequencing this clone to identify the 5′ coding region of the 66-kDa-protein gene and to confirm the 3′ coding region reported by Rosa et al. (32). An updated search of gene and protein databases using the published sequence of Rosa et al. (32) failed to identify significant homology to any known protein or nucleotide sequence. However, sequencing of the complete gene may yet reveal sequence homology or structural motifs that will provide clues as to the structural and functional role of the 66-kDa protein.

Interestingly, proteinase K treatment of the 66-kDa protein results in truncation rather than degradation of the entire protein as has been observed for OspA and OspB. This result may indicate that the N-terminal portion of the 66-kDa protein resides within the outer membrane or extends into the periplasmic space and thus is protected from proteolysis. The 66-kDa protein also differs from OspA and OspB in that the N terminus was unacylated and therefore available for direct protein sequencing (38). This feature indicates that the 66-kDa protein is not a lipoprotein. Rather, similarities in the outcome of protease susceptibility tests suggest that the 66-kDa protein may be analogous topologically to integral membrane proteins, such as the OmpA protein of *Escherichia coli* (31, 35). If this is true, then the 66-kDa protein can be envisioned to cross the outer membrane of *B. burgdorferi* several times and would possess several transmembrane domains. Indeed, hydropathic analysis of the partial gene sequence described by Rosa et al. (32) reveals at least four regions resembling transmembrane domains (data not shown). We believe that the 66-kDa protein may represent one of the rare outer membrane proteins or intramembranous particles of the *B. burgdorferi* molecular architecture model proposed by Radolf (30). Given the conserved nature of this protein among *Borrelia* spp. and its possible transmembrane location, one can speculate that the 66-kDa protein may function in signal transduction or nutrient transport. Immunoelectron microscopy using N-terminus-specific antibodies and C-terminus-specific antibodies would be necessary to confirm whether the 66-kDa protein represents a transmembrane protein.

We have identified and partially characterized a chromosomally encoded, surface-exposed protein that differs considerably from the known outer membrane proteins of *B. burgdorferi*. We are currently evaluating the ability of the 66-kDa protein to elicit protective immunity. Subsequent studies will attempt to establish the topological orientation of the 66-kDa protein in the outer membrane and ascertain its functional role in the physiology of *B. burgdorferi* and perhaps, the pathogenesis of Lyme borreliosis.

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