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Surface Exposure and Species Specificity of an Immunoreactive Domain of a 66-Kilodalton Outer Membrane Protein (P66) of the *Borrelia* spp. That Cause Lyme Disease

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A chromosomally encoded 66-kDa protein (P66) of *Borrelia* spp. that cause Lyme disease has previously been shown to be associated with the spirochetal outer membrane. A topological model of P66 predicts a surface-exposed fragment which links the N- and C-terminal intramembranous domains of the protein (J. Bunikis, L. Noppa, and S. Bergström, FEMS Microbiol. Lett. 131:139–145, 1995). In the present study, an immunogenic determinant of P66 was identified by a comparison of the immunoreactivities of different fragments of P66 generated either by proteolytic treatment of intact spirochetes or as recombinant proteins expressed in *Escherichia coli*. The immune response to P66 during natural infection was found to be directed against the predicted surface domain which comprises amino acids at positions 454 through 491. A sequence comparison revealed considerable polymorphism of the surface domains of P66 proteins of different Lyme disease-causing *Borrelia* species. Five sequence patterns of this domain were observed in the *B. garinii* strains studied. In contrast, sequences of the relevant part of P66 of the *B. afzelii* and *B. burgdorferi* sensu stricto isolates studied were identical within the respective species. In immunoblotting, 5 of 17 (29.4%) sera from North American patients with early disseminated or persistent Lyme disease reacted against P66 of *B. burgdorferi* sensu stricto B31. These sera, however, failed to recognize P66 of *B. afzelii* and *B. garinii*, as well as an analog of P66 in the relapsing fever agent, *B. hermsii*. In conclusion, the topological model of P66 is supported by the demonstration of an apparent surface localization of an immunoreactive domain of this protein. Furthermore, analogous to the plasmid-encoded borrelial outer surface proteins, the predicted surface-exposed portion of chromosomally encoded P66 appears to be antigenically heterogenous.

Lyme disease, the most common tick-borne infection in North America and Europe, is caused by *Borrelia burgdorferi* sensu lato species (4, 11, 12). Lyme disease is a phasic multisystemic disorder initiated by inoculation of a bacterium into the skin during a tick bite (for review see reference 36). In the majority of cases, intracutaneous migration of the spirochetes manifests itself in the frequent symptom of early infection, erythema chronicum migrans. Later, hematogenous spread of the borreliae may occur causing chronic dermatologic, neurologic, and arthritic complications.

Various pathogenetic events during different stages of infection are anticipated to be associated with the outer surface proteins (Osps) of *Borrelia* spp. that cause Lyme disease. These lipoproteins have been shown to assist adhesion to and invasion of the tissues by the spirochetes in vitro (14, 15, 38) as well as to promote infectivity of borreliae in vivo (32). Osps are also involved in the inflammatory processes by triggering the production of interleukins and are potent inducers of specific humoral and cellular immune responses (17, 21, 31, 37). These proteins may also contribute to the development of chronic Lyme disease by undergoing subtle antigenic changes which give rise to immunoevasive mutants (13, 19, 20, 33). OspA and OspB, however, are seldom expressed by borreliae in the mammalian host, and the biological activity of OspC, expression of which is turned on in the mammalian host, is not well characterized.

The structural and functional features of other borrelial outer membrane proteins are poorly understood. By proteolytic treatment and immunogold staining, a 66-kDa protein (P66) was recently shown to be associated with the outer membranes of the spirochetes and to be partially surface exposed (7, 9). Characteristically for a protein exported through the cytoplasmic membrane, sequence analysis of P66 reveals a leader peptide containing a signal peptidase I cleavage site. Furthermore, proteolysis of P66 in intact borreliae appears to occur at the C-terminal part of the protein, which features also a computer-predicted transmembrane helix. A model of P66 derived from the experimental data and computer analysis proposes a surface-exposed segment of the protein which links the N- and C-terminal intramembranous domains of P66 (9). It is intriguing that chromosomally encoded P66 has no homologs in current databases.

To address the functional importance of P66 we initially investigated the role of this protein in the host's immune response. We identified an immunogenic determinant of P66, which appeared to be associated with the predicted surface domain and to be specific to Lyme disease-causing *Borrelia* species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The designations and origins of the *Borrelia* strains used in this study are presented in Table 1. Borreliae were grown in BSK II medium (5), and the cells were harvested in late log phase by centrifugation at 3,000 × *g* for 20 min.

Escherichia coli DH5α and BL21 were used for transformation with recombi-

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TABLE 1. Designations and origins of the *Borrelia* strains studied

Strain or serotype[a]	Source		ATCC designation or reference
	Geographic	Biological	
B. burgdorferi			
B31	United States	*Ixodes scapularis*	ATCC 35210
HB19	United States	Human blood	7
Sh-2-82	United States	*I. scapularis*	34
B. afzelii			
ACAI	Sweden	Human skin	1
VS461	Switzerland	*I. ricinus*	29
U01	Sweden	Human skin	35
B. garinii			
Ip90	Asian Russia	*I. persulcatus*	25
NBS23A	Sweden	*I. ricinus*	8
NBS16	Sweden	*I. ricinus*	8
IUB18	Sweden	*I. uriae*	28
Mal01	Sweden	*I. uriae*	10
Mal02	Sweden	*I. ricinus*	10
Far01	Faroe Islands	*I. uriae*	10
Far02	Faroe Islands	*I. uriae*	10
Fis01	Iceland	*I. uriae*	10
Lab	Lithuania	*I. ricinus*	10

[a] Culture serotype of *B. hermsii* was used.

nant plasmids in DNA cloning and gene expression experiments, respectively. *E. coli* cells were grown in Luria-Bertani medium supplemented with carbenicillin (50 μg/ml) as required.

Preparation of bacterial proteins, SDS-PAGE, and immunoblotting. For the whole-cell protein preparations, bacteria were harvested from 15 ml of culture and washed twice with phosphate-buffered saline (PBS)–5 mM $MgCl_2$. After centrifugation, the pellet was resuspended in 0.1 ml of PBS, and the whole-cell lysate was obtained by boiling the bacteria for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Bacterial proteins were separated by SDS–12.5% PAGE. Subsequently, gels either were stained with Coomassie blue R-250 (Sigma, St. Louis, Mo.) or were subjected to immunoblotting. The proteins were transferred to a NitroBind membrane (Micron Separations Inc., Westboro, Mass.) by electroblotting at 0.8 mA/cm^2 for 1 h. Nonspecific binding was blocked by immersing the membrane in 5% bovine serum albumin (Sigma) in PBS containing 0.05% Tween-20 for 2 h. Primary and secondary antibodies were diluted with 2.5% bovine serum albumin in PBS–0.05% Tween-20. Patient sera were diluted 1:400 and investigated for the presence of specific immunoglobulin G antibodies. Incubations of the membrane with the antibodies for 1 h were followed by washing in PBS–0.05% Tween-20. 5-Bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) (Sigma) was the substrate for the alkaline phosphatase conjugate in the developing reaction.

Protease treatment of intact borreliae. Surface-exposed proteins of intact *B. burgdorferi* B31 and the relapsing fever agent, *Borrelia hermsii* C, were cleaved by proteinase K as previously described (7). Briefly, harvested and washed spirochetes were resuspended in PBS–5 mM $MgCl_2$ at a concentration of 2×10^9 cells per ml. To 950 μl of the cell suspension, 50 μl of either a solution of proteinase K in water (4 mg/ml) or distilled water was added. After incubation for 40 min at 20°C, 10 μl of phenylmethylsulfonyl fluoride (PMSF) (50 mg/ml in isopropanol; Sigma) was added and the cells were centrifuged and washed twice with PBS–5 mM $MgCl_2$. The pellet was resuspended in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, and 5 mM $MgCl_2$ and subjected to whole-cell protein extraction by being boiled in SDS-PAGE sample buffer. The efficiency of proteolysis was evaluated by monitoring the removal of OspA and OspB upon proteinase K treatment of intact spirochetes. The preservation of the integrity of the outer membrane during the procedure was suggested by the stability of flagellin, the major constituent of the borrelial periplasmic flagellum (7).

Cloning and expression of P66 gene fragments. PCR was performed on the P66 gene of *B. burgdorferi* B31 to obtain DNA fragments encoding different parts of P66. The plus and minus strand oligonucleotides used in the PCR contained created *Bam*HI and *Eco*RI restriction sites (underlined below), respectively. The plus strand oligonucleotides targeted the P66 gene at positions 1395 through 1423 (primer 1, 5′-TGGATTAGGATCCATAACATCTATCGGTC-3′) and 1534 through 1566 (primer 2, 5′-CCTAATCTGACATTTGGATCCGCAATG AAACTC-3′) according to the previously published sequence (9). The minus strand oligonucleotides were directed to positions 1563 through 1534 (primer 3, 5′-TTTCATTGCGAATTCAAATGTCAGATTAGG-3′) (9) and 1877 through 1849 (primer 4, 5′-CCAAAGGAATTCTTTTGCTGTTAGCTTCC-3′) (9). By using primers 2 and 4 in the PCR, we obtained the DNA sequence that encodes a C-terminal intramembranous fragment of P66 (10.8 kDa) (Fig. 1). The DNA fragment for the recombinant surface-exposed domain of P66 (5.3 kDa) was generated by PCR with primers 1 and 3. Finally, primers 1 and 4 amplified the DNA sequence for the recombinant peptide (16.2 kDa) comprising the surface-exposed and the C-terminal intramembranous domains of P66 (Fig. 1).

After digestion with endonucleases, the PCR products were ligated into the pGEX2T expression vector (Pharmacia Biotech, Uppsala, Sweden) in frame with the plasmid-encoded glutathione *S*-transferase (GST) gene (22). The recombinant plasmids were used to transform *E. coli* BL21. Expression of the fusion proteins was induced in the culture by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) to a final concentration of 0.1 mM. The fusion proteins were identified by SDS-PAGE and immunoblotting with patient sera. In addition, the N-terminal amino acids of the recombinant fragments of P66 that remained after thrombin cleavage of the fusion protein were sequenced.

PCR amplification of DNA fragments encoding the surface-exposed domain of P66 was also performed on DNA from several *Borrelia* species isolates (Table 1). The PCR products were ligated into the pT7Blue T-vector (Novagen, Madison, Wis.) and transformed into *E. coli* DH5α. Positive clones were sequenced by the dideoxy chain termination method, with α-^{35}S-labeled dATP (Pharmacia Biotech T7 sequencing kit). Sequences were analyzed with the University of Wisconsin Genetics Computer Group Sequence Analysis and PC-Gene computer software.

Serum samples. The battery of reference sera and clinical summaries of 40 well-defined Lyme disease cases from the United States were provided by Barbara Johnson of the Centers for Disease Control and Prevention, Fort Collins, Colo. The Lyme disease cases were clinically classified into the stages of early localized or disseminated and persistent infection according to the guidelines described elsewhere (36).

RESULTS

Generation and immune reactivity of different fragments of P66. To characterize the possible antigenic determinants of P66, the immunoreactivities of different regions of P66 were compared. As has been shown previously (7, 9), upon proteinase K treatment of intact borreliae, P66 is cleaved and an approximately 50-kDa fragment of this protein sequesters within the cells (see also Fig. 2A). The identity between the N-terminal amino acid sequences of the full-length and trun-

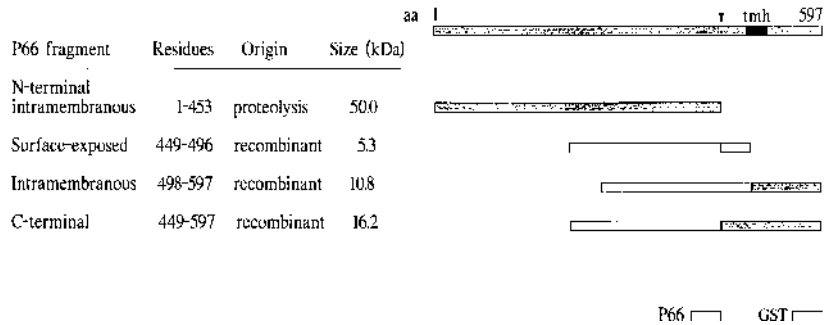

FIG. 1. Schematic presentation of the P66 structural elements generated for the immunoreactivity study. An arrowhead indicates the predicted proteolytic cleavage site at glycine, position 453, of the mature P66 of *B. burgdorferi* B31 (9). A computer-predicted transmembrane helix (tmh) comprises amino acids at positions 492 to 517. Different fragments of P66 are expressed as protein fusions with pGEX2T-encoded 26-kDa GST.

cated P66 (9) indicates the presence of proteolytic cleavage site(s) at the C terminus of the protein. To define the site of proteolysis and, consequently, to infer the start of the surface-exposed part of P66, we attempted to obtain the C-terminal amino acid sequence of the truncated P66. This attempt, however, was unsuccessful because of a possible block at the C-terminal amino acid of the truncated P66. Therefore, the candidate C-terminal residue had to be predicted by computer from the sequence of P66 (9) by cumulating the molecular weights of amino acids that make up a peptide with a molecular mass of 50 kDa. Thus, glycine at position 453 of a mature P66 of *B. burgdorferi* B31 was predicted to be at the C terminus of the N-terminal intramembranous portion of P66. Although this approach allows certain inaccuracy, we believe it was adequate for the designing of the recombinant fragments of P66 which were subsequently characterized in the immunoreactivity study (Fig. 1). Another computer-predicted structural element of P66 which assisted the design of these fragments is a transmembrane helix which starts 38 residues downstream from the aforementioned glycine and comprises amino acids at positions 492 to 517 (Fig. 1) (9). The transmembrane helix is suggested to position the P66 into the outer membrane and to be a part of the C-terminal intramembranous domain of P66 (Fig. 1) (9). It has been proposed previously that the N-terminal and C-terminal intramembranous fragments of P66 are linked by the predicted surface-exposed loop structure (9).

The immunoreactivities of different fragments of P66 were compared by using the P66-positive sera from patients with early disseminated and persistent Lyme disease by immunoblot analysis. Neither the proteolytically generated N-terminal fragment nor a recombinant C-terminal intramembranous fragment of P66 of *B. burgdorferi* B31 reacted with these sera, which otherwise showed strong reactivity with full-length P66 (Fig. 2). In contrast, the patient sera recognized recombinant fusion proteins which contained the surface-exposed domain either alone or in combination with the C-terminal intramembranous domain (Fig. 1). In addition, the polyclonal monospecific rabbit antiserum against P66 (9) reacted with the N-terminal intramembranous fragment but did not react with the recombinant C-terminal intramembranous domain or with a native C-terminal intramembranous fragment in the lysate of spirochetes treated with proteinase K (data not shown).

Interestingly, the recombinant fusion proteins of P66 demonstrated different solubilities upon sonication of the *E. coli* cells. The recombinant fusion protein containing the surface-exposed domain of P66 appeared to be soluble in the sonicate. In contrast, the recombinant C-terminal fragment as well as the C-terminal intramembranous portion alone formed insoluble aggregates (inclusion bodies).

Species specificity of the immunoreactive domain of P66. To examine the antigenic polymorphism of the immunoreactive fragment of P66, segments of the genes encoding the surface-exposed domains of the proteins from different Lyme disease *Borrelia* strains were cloned and sequenced. A comparison of the deduced amino acid sequences of the surface-exposed fragments of P66 revealed significant sequence polymorphism (Fig. 3). The identity between sequences of the full-length P66 in Lyme disease-causing *Borrelia* species is 91.5 to 93.8% (9), whereas the surface-exposed domains appeared to be 71.1 to 81.6% identical. Interestingly, the sequences of different *Borrelia afzelii* and *B. burgdorferi* sensu stricto isolates were identical within the respective species, whereas the *Borrelia garinii* isolates diverged into five sequence patterns. Three blocks of conserved amino acids were, however, recognized in the sequences of all *B. garinii* isolates (Fig. 3). P66 surface-exposed domain sequence types 1 to 3 of *B. garinii* corresponded to individual OspA serotypes previously demonstrated for these strains (10). Moreover, strains of sequence types 4 and 5 corresponded to OspA serotypes 3 and 6, respectively, in the aforementioned study.

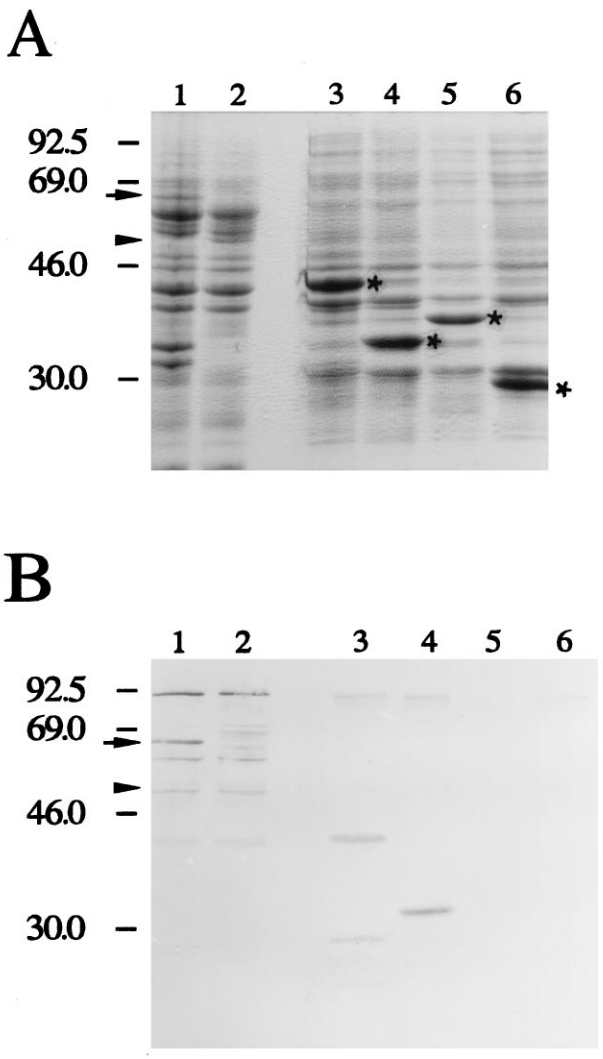

FIG. 2. Origin and immunoreactivity of different domains of P66. (A) Proteins of intact and proteinase K-treated *B. burgdorferi* B31 (lanes 1 and 2) and IPTG-induced *E. coli* BL21 transformants (lanes 3 to 6) were resolved by SDS–12.5% PAGE and Coomassie blue stained. Preparations of *E. coli* produced recombinant GST alone (lane 6) or GST fused to one of the following: the surface-exposed domain followed by the C-terminal intramembranous portion of P66 (lane 3), the surface-exposed domain of P66 (lane 4), or the C-terminal intramembranous portion of P66 (lane 5). Recombinant GST and GST fusions in *E. coli* lysates are indicated by stars. (B) Bacterial proteins loaded in the same order as those shown in panel A were separated by SDS–12.5% PAGE, blotted onto a nitrocellulose membrane, and reacted with serum from a North American patient with early disseminated Lyme disease (erythema chronicum migrans and Bell's palsy). The positions of full-length and truncated P66 are shown by arrows and arrowheads, respectively. Molecular masses in kilodaltons are on the left.

To validate the experimental data, a computer search was performed to look for a potential antigenic determinant in the P66 sequence. The secondary-structure analysis of the full-length P66 predicted a segment with the highest peak of flexibility exactly within the surface-exposed domain of the protein. In the P66 sequence of *B. burgdorferi* B31 this segment included amino acids at positions 479 to 485 (Fig. 3). Flexible segments were also predicted at similar positions in the P66

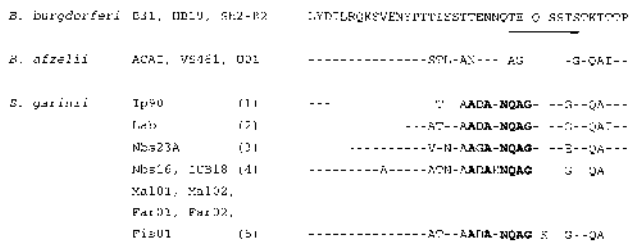

FIG. 3. Amino acid sequence comparison of the predicted surface-exposed domains of P66 in Lyme disease-causing *Borrelia* species. *B. burgdorferi* sensu stricto B31, *B. afzelii* ACAI, and *B. garinii* Ip90 sequences have been published previously (9). Deduced amino acid sequences are presented. The *B. burgdorferi* B31 sequence comprises amino acids at positions 454 to 491 of the mature peptide (9). The computer-predicted flexible segment is underlined. *B. garinii* displays five different sequence types, as indicated by the numbers in parentheses; the conserved amino acids in the *B. garinii* isolates are in boldface. The following accession numbers have been assigned to the P66 gene sequences by EMBL: B31, X87725; ACAI, X87726; Ip90, X87727 (9).

sequences of *B. afzelii* ACAI and *B. garinii* Ip90 despite marked sequence diversity in this locus.

Reactivity of Lyme disease patient sera with P66. The reactivities of Lyme disease patient sera with P66 of *Borrelia* strains from different species were examined by immunoblot analysis. Sera from North American patients with Lyme disease reacted with P66 of *B. burgdorferi* B31 but failed to recognize P66 of *B. afzelii* ACAI and *B. garinii* Ip90 (Fig. 4). Furthermore, these sera did not react with the analog of P66 in *B. hermsii* C (Fig. 4), which has been identified in this *Borrelia* species by proteolytic treatment of intact spirochetes (data not shown).

In total, 20% of selected Lyme disease patients from the United States developed an immunoglobulin G response against P66 of *B. burgdorferi* B31. Anti-P66 reactivity tended to be greater in patients with early disseminated or persistent infection. None of the five control sera showed reactivity with P66.

DISCUSSION

Previous studies have demonstrated the association of a 66-kDa protein, P66, with the outer membranes of Lyme disease-causing *Borrelia* species (7, 9, 30). The proteolytic cleavage pattern and computer predictions based on the sequence data of P66 led us to propose a topological model of the protein (9). It is suggested that two intramembranous domains are linked by a surface-exposed and, therefore, protease-accessible portion of the protein. In the present study, the suggested topology of P66 was supported by defining an immunogenic determinant of the protein.

Several experimental observations indicate that the antigenicity of P66 during natural infection is associated with the predicted surface-exposed part of the protein. In immunoblot analysis, P66-positive sera from Lyme disease patients reacted only with the recombinant fragments of P66 which contained the surface domain. In contrast, proteolytically generated N-terminal and recombinant C-terminal intramembranous domains of P66 did not react with patient sera. Poor antigenicity of the C-terminal intramembranous domain of P66 is also suggested by the hydrophobic nature of the recombinant proteins containing this domain. Moreover, a polyclonal antiserum against P66 fails to detect the native C-terminal intramembranous domain in the spirochetal lysate after proteolytic treatment. These results taken together indicate that the C-terminal intramembranous portion of P66 is weakly immunogenic both under natural and experimental conditions.

Our experimental data showing that the surface-exposed domain is immunogenic were supported by the prediction of a flexible segment within the sequence. Segmental flexibility is thought to correlate with the locations of continuous epitopes in proteins (40). The position of the flexible segment was preserved in all Lyme disease-causing *Borrelia* species, despite considerable heterogeneity in this region. This suggests that antigenicity is a conserved feature of the surface-exposed domain of P66.

A comparison of the deduced amino acid sequences of the surface-exposed domains of P66 of the three Lyme disease-causing *Borrelia* species studied revealed considerable sequence polymorphism. The *B. garinii* strains included in this study, in contrast to the *B. afzelii* and *B. burgdorferi* sensu stricto strains, diverged into five sequence types, which supports the accepted view of the exceptional genetic and phenotypic heterogeneity of this Lyme disease-causing *Borrelia* species (41, 42). The sequences of the surface-exposed portions of P66 in *Borrelia* strains of the three species were 70 to 80% identical, which is comparable to the heterogeneity exhibited by the plasmid-encoded Osps (23, 24). Amino acid sequence identity of the full-length P66 in Lyme disease-causing *Borrelia* species exceeds 90% (9), which corresponds to the degree of diversity demonstrated by other chromosomally encoded borrelial proteins, e.g., flagellin (27). Thus, irrespective of genetic origin, the surface-exposed immunogenic determinants of Lyme disease-causing *Borrelia* species are likely to show significant sequence variability. Positive selection factors, including the immune responses of mammals and adaptions to different environments, are suggested to play a role in generating antigenic diversity of Osps. The heterogeneity of the surface-exposed domain of P66 compared with that of other structural elements of this protein indicates that similar mechanisms could possibly be responsible also for the evolvement of different antigenic variants of P66. Moreover, the sequence types of the surface-exposed domains of P66 and OspA serotypes correlated in *B. garinii* strains, suggesting a common source of the selective pressure experienced by these two proteins.

The immune response against P66 during natural infection appears to be specific to the Lyme disease-causing *Borrelia* species because sera from North American patients with Lyme disease recognize P66 only of *B. burgdorferi* B31, the type strain in the United States. P66 proteins of *B. afzelii* and *B. garinii*

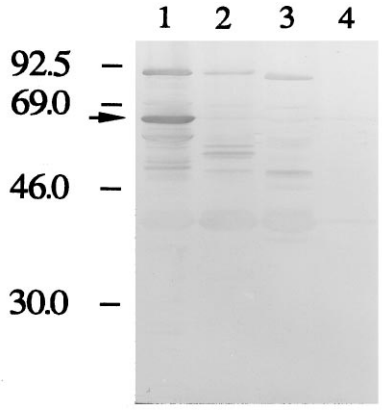

FIG. 4. Species specificity of the immune response against P66. Borrelial proteins were separated by SDS–12.5% PAGE, blotted onto a nitrocellulose membrane, and reacted with serum from a patient as described in the legend to Fig. 2. Preparations were loaded as follows: Lane 1, *B. burgdorferi* B31; lane 2, *B. afzelii* ACAI; lane 3, *B. garinii* Ip90; lane 4, *B. hermsii*. The arrow indicates the position of P66.

strains, as well as the analog of P66 in *B. hermsii* C, were not detected by these sera. P66 was able to trigger detectable serum immunoglobulin G levels in 30% of selected patients with early disseminated or persistent Lyme disease. This percentage is similar to that associated with the reactivity seen against specific borrelial immunogens OspA and OspB (18, 26).

Several studies have demonstrated immune responses and clinical signs of Lyme disease that are *Borrelia* species specific (2, 3, 6, 17, 39, 43). Because of the established polymorphism, borrelial Osps are believed to account for some of these differences (41, 42). The surface exposure and antigenic heterogeneity of P66 suggest that this protein may also be involved in the species-specific immunological and pathogenetic mechanisms of Lyme disease.

In conclusion, the results of this study support the topological model of P66 by demonstrating the surface location of an immunoreactive domain of this protein. Analogous to the plasmid-encoded Osps, the surface-exposed portion of chromosomally encoded P66 is specific to the Lyme disease-causing *Borrelia* species.

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