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The 93-Kilodalton Protein of *Borrelia burgdorferi*: an Immunodominant Protoplasmic Cylinder Antigen

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Using immunoblots, we identified proteins of *Borrelia burgdorferi* recognized by sera from 62 patients with either acute or chronic Lyme disease. In all groups studied, the 41-kDa flagellar protein and a relatively minor 93-kDa protein (p93) were the most commonly recognized antigens in patients with acute and chronic disease due to *B. burgdorferi*. A murine monoclonal antibody (MAb 181.1) was developed against p93, and the antigen was detected by immunoblot analysis in four European and American strains of *B. burgdorferi*. On two-dimensional gel electrophoresis, p93 had an apparent pI of 6.8. Immunoelectronmicroscopy with MAb 181.1 demonstrated that p93 is located within the protoplasmic cylinder compartment of the organism. The gene encoding p93 was retrieved from a phage expression library. The derived amino acid sequence of p93 confirmed chemical characterization of the antigen, including its amino-terminal peptide sequence. The derived amino acid sequence predicted it to be predominantly alpha helical. A prominent antigenic domain located at the carboxy portion of the protein was recognized by human and rabbit polyclonal antisera and human (MAb D4) and mouse (MAb 181.1) MAbs.

Lyme borreliosis is a tick-borne infectious disease that displays a wide array of clinical manifestations (42). The earliest sign of infection, erythema migrans, occurs in approximately two-thirds of acutely infected patients (66). Shortly after infection, the organism may disseminate throughout the body and give rise to a variety of clinical manifestations of infection, including multiple areas of erythema migrans, meningitis, meningoencephalitis, painful radiculitis, cranial neuritis, and arthritis (5–7, 12, 32, 60, 66, 71). In many untreated cases, the acute infection is self limiting and becomes latent only to recrudesce later in life as a chronic infection involving the joints, heart, nervous system, or skin (3, 29, 67, 70). The symptoms associated with the chronic infection may be vague and not associated with demonstrable clinical signs of disease. It is unclear whether the vague symptoms of late disease can be attributed to an actual ongoing infection or whether they result from some other pathogenic mechanism.

The etiologic agent of Lyme borreliosis, *Borrelia burgdorferi*, was isolated and cultivated in 1982 (16). Isolation and culture of this organism from the obviously infected host are extremely difficult (4, 11, 68, 69); therefore, diagnosis of active infection has been dependent on the demonstration of an immune response to *B. burgdorferi* in an appropriate clinical setting. The serologic tests that have been performed are relatively insensitive. As many as 50% of patients with erythema migrans may have a negative antibody titer by the standard immunofluorescence assay or enzyme-linked immunosorbent assay (ELISA) (1, 22, 49, 51, 53, 62). Furthermore, patients with evidence of chronic infection have been reported to have negative serum antibody titers (23, 35, 41, 72). In addition, cross-reactive antibodies to *B. burgdorferi* have been found in patients with various infectious and noninfectious diseases as well as in the healthy general population (9, 34, 44, 47, 48). This has led to a high background in the immunofluorescent assay and the ELISA. Consequently, it becomes necessary to increase specificity of these tests by further dilution of the sera, and this results in loss of sensitivity of the test.

The lack of specificity of the serological tests is due to cross-reactive epitopes found on antigens of *B. burgdorferi*. During acute infection with *B. burgdorferi*, the antibody response is slow to evolve, and the full repertoire of antigens is not recognized for months to years after infection (21). Early in the infection, the antibody response is directed against the 41-kDa flagellum-associated antigen. This protein has significant homology to the 33-kDa flagellar protein of *Treponema pallidum* (27, 46). Humoral response to other antigens, including the 20- to 22-, 39-, 60-, 66-, 68-, 71-, and 73-kDa proteins, gradually develops (9, 21, 78). It is significant to note that the 60-, 66-, and 68-kDa proteins have amino-terminal sequence homology and immunologic cross-reactivity to the GroEL family of heat shock proteins, and the 71- and 73-kDa proteins have amino-terminal sequence homology and immunologic cross-reactivity to the DnaK family of heat shock proteins (17, 18, 30, 43). Cross-reactivity between these prominent antigens and heat shock proteins from unrelated organisms may further explain the high level of immunologic cross-reactivity noted. By contrast, the OspA and OspB proteins are specific for *B. burgdorferi*, but the humoral response to these antigens occurs in only a small percentage of patients (9, 21, 78). A number of investigators have also noted a prominent immune response to proteins in the 83- to 100-kDa molecular mass range in patients with both early and late Lyme disease (21, 59, 76, 79). In this study, we have identified and immunologically and biochemically characterized a dominant immunogen of *B. burgdorferi*. We have isolated and sequenced clones containing this gene retrieved from a lambda gt11 expression library, determined the derived amino acid sequence of the protein,

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expressed this protein in T7 and pATH3 expression systems, and identified a dominant antigenic domain.

MATERIALS AND METHODS

Bacterial strains. Four isolates of *B. burgdorferi*, 297 (28) and IP-2 (originally isolated from the cerebrospinal fluid of patients in Connecticut and France, respectively) obtained from Russell Johnson, University of Minnesota, Minneapolis, and the Arc strain (originally isolated from skin biopsy of a patient with erythema migrans [13] in New York) obtained from Bernard Berger (Southampton Hospital, Southampton, N.Y.) were grown in Barbour-Stoener-Kelly II (BSK II) medium (8) at 33°C for 3 days to a concentration of 5×10^7 organisms per ml. *Borrelia hermsii* (HS1 strain), *Borrelia parkeri*, *Borrelia turicatae*, and *Borrelia coriaceae* (obtained from Tom Schwan, Rocky Mountain Laboratories, Hamilton, Mont.) were grown in a similar manner. Spirochetes were centrifuged at 10,000 × *g* at 10°C for 15 min, washed three times in 200 ml of 0.01 M phosphate-buffered saline (PBS; pH 7.2), enumerated by dark-field microscopy, and treated as previously described (46). *T. pallidum* and *Treponema denticola* (ATCC 35022) antigens were generously provided by Sheila Lukehart, University of Washington, Seattle, and Thomas MacNamara, State University of New York at Stony Brook, Stony Brook.

PAGE. Spirochete preparations were dissolved in a sample buffer that contained 0.5 M Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 20% glycerol, 10 mM EDTA, 0.001% bromphenol blue, and, for reducing gels, 5% 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) and incubated at 95°C for 1 min. After solubilization, the suspension was centrifuged at 10,000 × *g* for 1 min to remove nonsolubilized material, and the supernatant was used for SDS-polyacrylamide gel electrophoresis (PAGE) or aliquoted and frozen at −70°C. Protein concentration was determined by the Bradford method (15). Preparations (25 μg per lane) dissolved in sample buffer were electrophoresed in a discontinuous SDS–0.1 to 10% polyacrylamide gel (Bio-Rad dual-slab cell) at 35 mA per gel with the Laemmli buffers (40). The gel was stained and fixed with 0.15% Coomassie blue–10% acetic acid–50% ethanol and then destained with 30% isopropanol–10% acetic acid.

Amino acid analysis and protein microsequencing. For two-dimensional gel electrophoresis, the spirochetes were solubilized in a sample buffer containing 9.4 M urea, 2% ampholytes, 2% dithiothreitol, 20 mM methylamine (pH 7.6), and 15 mM thioglycolic acid (77). Isoelectric focusing in the first dimension was followed by SDS-PAGE in the second dimension as described by O'Farrell et al. (55, 56) and modified by Anderson and Anderson (2) using two-dimensional electrophoresis. Isoelectric focusing was performed in polyacrylamide tube gels containing 9.2 M urea, 5% ampholytes (pH 3.5 to 10.0; LKB Pharmacia, Piscataway, N.J.), 4% acrylamide–bisacrylamide (30:1.8), 2% Nonidet P-40 (Sigma). Iso-tube gels were prefocused at 200 V for 1 h prior to the loading of the samples. Subsequent to sample loading, a constant voltage was applied for 18 h at 511 V and then for 1 hour at 800 V. Immediately after focusing was completed, the pH gradient was measured directly with a flatbed pH electrode (Microelectrode, Inc., Londonderry, N.H.). Separation in the second dimension was done with SDS–12.5% polyacrylamide slab gels.

Proteins separated by two-dimensional gel electrophoresis were electroblotted onto Immobilon-polyvinylidene difluoride (PVDF) (Millipore) (38). The blot was stained with amido black, destained with 100% methanol, and rinsed with deionized water. The blotted p93 (93-kDa) protein was used for amino acid analysis. The sample was subjected to acid hydrolysis with 6 N hydrochloric acid for 24 h. The amino acids were extracted by using standard procedures (52). Analysis was performed with a Pico Tag amino acid analyzer (Waters Chromatography Division, Milford, Mass.) (19). Amino acids were quantitated by peak area comparison to a 250-pmol standard assay of 16 amino acids (19). To determine whether cysteine residues were present, eluted p93 samples that had been dialyzed in water were first subjected to oxidation by using performic acid prior to acid hydrolysis. The oxidation of cysteine was performed as previously described (19, 26). Amino-terminal sequences were determined on the blotted p93 with a gas phase or pulse-liquid microsequencer (model 470A/475A; Applied Biosystems) coupled to an on-line high-performance liquid chromatograph (model 120A; Applied Biosystems) by using the manufacturer's standard protocols and programs.

Immunoblot studies. Proteins separated by one- or two-dimensional gel electrophoresis were transferred to Immobilon-PVDF (Millipore) as described by Towbin et al. (75). Efficiency of transfer was determined by amido black staining. Unreacted protein-binding sites were blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 0.15 M NaCl, 20 mM Tris hydrochloride [pH 7.4]) for 2 h at room temperature. The blots were washed in TBS three times and incubated overnight with sera from humans or rabbits containing anti-*B. burgdorferi* antibody at dilutions of 1:100 or 1:1,000, respectively. The rabbit serum was prepared as previously described (46). Blots were then washed in TBS containing 2% BSA and incubated with 500,000 cpm of ^{125}I-labeled staphylococcal protein A (specific activity, 65 mCi/mg; 0.1 μCi/ml; New England Nuclear, Philadelphia, Pa.) or alkaline phosphatase-conjugated goat anti-rabbit sera (Sigma) for 2 h at room temperature. Following washing, individual blots were autoradiographed for various periods of time or alkaline phosphatase activity was detected by using nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate (Kirkegaard and Perry, Richmond, Calif.) as directed by the manufacturer.

MAb. Murine monoclonal antibodies (MAbs) were developed and isotyped as previously described (37). One clone (MAb 181.1) was selected for this study because of reactivity to the 93-kDa protein on immunoblot analysis. This clone was subcloned by limiting dilution, and the culture supernatants were used for analysis. Another previously described MAb (51) (MAb 184.1) reactive to the 31-kDa OspA protein was selected for control experiments. A human MAb, MAb D4 (52), with reactivity to the 93-kDa protein was generously provided by David Volkman, State University of New York at Stony Brook.

Preparation and screening of a *B. burgdorferi* expression library. The *B. burgdorferi* lambda gt11 expression library was constructed as described by Huynh et al. (36). The B31 strain of *B. burgdorferi* was grown to the mid-log phase. Total genomic DNA was isolated and sheared to generate fragments of 1 to 4 kb by repeated sonication. The genomic DNA was then methylated with *Eco*RI methylase (New England BioLabs, Inc., Beverly, Mass.). The sheared ends were made flush with T4 DNA polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and *Eco*RI linkers were ligated to the blunt ends. After digestion with *Eco*RI, the excess linkers were removed by gel electrophoresis. The processed DNA was fractionated on 1% agarose,

TABLE 1. Oligonucleotides used in this study

Oligonucleotide no.	Sequence
1	5'GGAGCAGCTTAAAGAAACTGGTGATGA3'
2	5'CAATAGTTTCTGTTATATTGGAATCAC3'
3	5'CTCCCCAATACCCACTATTTGTT3'
4	5'CATCACCATGGGAGTGCAAGAGAAGTTGATAGG3'
5	5'CGCGGATCCTTACTTAACTTTTTTTAAAGTATTTAC3'
6	5'GGAATTCCAATATTGAGTCTGACATTGATATTGAC3'
7	5'GGAATTCCGAGGTTGAGAAATTAGATAAGATTTTC3'
8	5'GGAATTCCTTAAATACTGGTGTTAGGCTTAAAGAA3'
9	5'GGAATTCCATTTTAGTAGCTGTTAGGGATAAAGAT3'
10	5'CGGATCCATCAATAAGTTGAAGAGTTCCTAAATT3'
11	5'CGGATCCCATTTTAGAATCAACATAAAGAGATGA3'
12	5'CGGATCCTTACTTAACTTTTTTTAAAGTATTTAC3'

and 1- to 4-kb fragments were recovered by standard extraction procedures. This DNA was ligated into *Eco*RI-digested dephosphorylated lambda gt11 arms (Bethesda Research Laboratories), packaged with the Packagene system (Promega Biotech), and plated onto *Escherichia coli* Y1090.

The library was screened essentially as previously described (36, 81). Briefly, the library was plated on Luria-Bertani agarose with Y1090 and incubated at 42°C for 4 h. Nylon filters saturated with 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were overlaid on the plates. The plates were then incubated for an additional 2 to 3 h at 37°C, after which the filters were gently removed and rinsed in TBS. Nonspecific protein-binding sites were blocked by incubating the filters overnight at 4°C with TBS containing 5% nonfat dry milk. The filters were then incubated with MAbs diluted in TBS with 0.5% BSA. The filters were then washed three times with TBS–0.05% Tween 20 containing 0.05% sodium azide. Antibody binding was detected by incubating the filters for 2 h with rabbit anti-mouse antibodies conjugated to alkaline phosphatase (Sigma). Alkaline phosphatase activity was detected by using the NBT-BCIP substrate (Kirkegaard and Perry) as directed by the manufacturer.

Sequence analysis of the p93 genomic clone. Inserts from the immunoreactive lambda gt11 clones were retrieved by polymerase chain reaction (PCR) with lambda gt11 primers obtained from New England BioLabs. Amplified fragments were cloned into pVZ1, and a set of overlapping clones was sequenced (63). This set of overlapping clones was used to determine the entire sequence by inverse PCR (54). All the clones contained the same carboxy-terminal coding sequence terminated by a stop codon but did not contain the 5' end of the gene. We therefore performed two rounds of inverse PCR to obtain the 5' end. *B. burgdorferi* DNA was digested with *Bst*YI and ligated at a DNA concentration of 1 μg/ml. This was followed by 25 cycles of PCR (94°C for 30 s; 55°C for 1 min; 72°C for 2 min) with the following primers, which were determined from our DNA sequence data and are listed in Table 1: oligonucleotide 1, beginning at position 1095 in Fig. 7, and oligonucleotide 2, beginning at position 787 in Fig. 8. The amplified DNA was purified from 2% agarose gels, subcloned into pVZ1, and sequenced. A second round of inverse PCR was done to determine the remainder of the sequence by *Hin*dIII digestion, circularization, and PCR amplification by using the first primer listed above and oligonucleotide 3, beginning at position 183 in Fig. 7.

Preparations of p93 expression clones. The p93 open reading frame between bp 61 and 2103 was retrieved from B31 genomic DNA by amplifying the coding sequence by PCR using oligonucleotides 4 and 5 (Table 1; see Fig. 7), which were designed to contain terminal *Nco*I and *Bam*HI restriction sites, respectively. The resultant fragment was cleaved with *Nco*I and *Bam*HI and ligated to the T7 expression vector, pET7HIS.2 (73), which had also been cleaved with *Nco*I and *Bam*HI to create p93Δ20, a protein lacking the 20-amino-acid N-terminal leader sequence. Subclones of the p93 gene were prepared as TrpE fusion proteins in pATH3 (39) following our previously described methods (64). Five deletion constructs of p93 were constructed by PCR amplification by using a series of oligonucleotides indicated in Fig. 7 and Table 1. The oligonucleotide primers contained an *Eco*RI site at the 5' end and a *Bam*HI at the 3' end to facilitate cloning directionally into pATH3. The pATH constructs are referred to as E1 to E5.

Preparation of recombinant *B. burgdorferi* lysogens. Lambda lysogens of recombinant lambda gt11 clones were made as described by Huynh et al. (36). The lysogen cultures were grown in LB medium at 32°C to an optical density at 550 nm of 0.5, after which the temperature was shifted to 42°C for 20 min. IPTG was added to the culture at a final concentration of 10 mM and incubated at 37°C for an additional hour. The organisms were then centrifuged at 10,000 × *g* for 1 min and washed twice in PBS. After the final wash, the cells were resuspended in PBS, sonicated, and frozen at −20°C for later Western blot (immunoblot) analysis.

Northern blot analysis. RNA was prepared as described previously (74). Transfers of mRNA species from formaldehyde containing agarose gels to nylon membranes (Biodyne A, 1.2-μm pore size; ICN Pharmaceuticals Inc., Irvine, Calif.) were performed as described previously (50). Nucleic acid probes were radiolabeled with [α-^{32}P]dATP by random priming (25).

Pulsed-field gel electrophoresis. Agarose blocks containing B31 were prepared essentially as described by Fedows and Barbour (24) with minor modifications. Spirochetes were grown to a cell density of 10^8 cells per ml. Cells were harvested by centrifugation at 8,000 × *g* for 15 min at 20°C and then resuspended in 10 mM Tris (pH 7.5)–150 mM NaCl (TN buffer) at 37°C. An equal volume of molten 1% agarose (FMC BioProducts, Rockland, Maine) in TN buffer was added to the cell suspension, and the mixture was poured into acrylic casting wells. After the blocks solidified, they were first immersed in the lysis solution, which in its complete form was 50 mM Tris (pH 8.0)–50 mM EDTA–1% SDS–1 mg of proteinase K (Boehringer Mannheim) per ml, and then incubated at 50°C for 16 to 24 h. The DNA concentration in the melted blocks was determined by microfluorimetry (Hoefer) with calf thymus DNA in agarose as a standard. Agarose blocks were stored in 10 mM Tris (pH 8.0)–1 mM EDTA (TE buffer) at 4°C until use. Electrophoresis was performed with the Bio-Rad CHEF-DRII system. The DNA blocks were washed in TE buffer and then loaded into 1% agarose gel with 0.5× TBE buffer (1× TBE is 45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA) set at a pulse time of 60 s at 200 V for 18 h. Yeast chromosomes (strain YNN 295) from Bio-Rad were used as molecular weight markers.

Electron microscopy. Electron microscopy was performed on whole untreated organisms and a fraction of the organisms after treatment with *N*-Sarkosyl. For the latter, 10^{10} cells were suspended in 10 ml of TE with 0.2% Sarkosyl. Three-microliter volumes of live *B. burgdorferi* (10^6 organ-

isms per ml) or the Sarkosyl-insoluble cylinder preparation (3 μg/μl) suspended in PBS were placed on 0.2% Formvar-coated and carbon-reinforced 200-mesh copper grids. Samples were allowed to absorb for 30 min at room temperature. Excess fluid was removed by absorption with a Whatman filter, the grids were washed with TBS, and protein-binding sites were blocked with 0.1% BSA with or without 0.1% Tween 20. Undiluted MAb supernatants were layered onto grids and incubated for 30 min. Unbound antibody was washed off with PBS. MAb binding was detected with goat anti-mouse antibody bound to 10-nm-diameter colloidal gold particles. After the final wash with distilled water, the preparations were then negatively stained with 2% uranyl acetate (pH 3.9). The samples were then air dried and examined with a Hitachi 11E-1 electron microscope.

Immune precipitation of recombinant expression clones. Recombinant subclones containing TrpE fusion proteins were induced with indoleacrylic acid as previously described (64). Following a 2-h induction, cells were incubated with 100 μCi of TranSlable (2 μCi/μg; ICN) for 10 min. Bacteria were washed three times in PBS and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 1% Nonidet P-40, 1.0% deoxycholate, 0.1% SDS). Cell lysates were precleared by incubation with 50 μl of Pansorbin (Calbiochem) at 4°C for 30 min and centrifuged for 1 min at 12,000 × *g*. Antibodies (6 μl of rabbit serum and 100 μl of MAb supernatants) were added and incubated on ice for 1 h. Immune complexes were precipitated by the addition of 50 μl of Pansorbin. Samples were suspended in SDS sample buffer, electrophoresed, and autoradiographed.

Human subjects. Sixty-two patients with definite Lyme disease were studied. Thirty-seven had erythema migrans, 12 of whom had a localized rash without systemic evidence of infection and 25 of whom had evidence of disseminated infection with multiple erythema migrans lesions, meningitis, hepatitis, or carditis. Twenty-five patients had chronic infection with a longer-than-4-month history of either (i) arthritis characterized by pain when the affected joint was moved, periarticular soft tissue swelling, synovial thickening, or small effusions or (ii) peripheral neuropathy. Sera of all patients studied were reactive to *B. burgdorferi* both in a standardized ELISA (23) and by immunoblot analysis (23). The control group of subjects consisted of seven healthy adults with no symptomatic or serological evidence of Lyme disease, five patients with known rheumatoid arthritis and seven patients with systemic lupus erythematosis.

RESULTS

Immune response to the 93-kDa protein. To determine the antigens recognized by sera of patients with Lyme borreliosis, immunoblot analysis was performed with the sera of 62 patients with clinical and serological evidence of Lyme borreliosis. All patients had a demonstrable antibody response as determined by a positive value in an anti-*B. burgdorferi* ELISA and a positive immunoblot analysis. Thirty-seven patients had acute disease manifested by erythema migrans, and 25 had prolonged (>6 months) disease manifested by rheumatological or neurological signs of chronic recurrent or chronic progressive disease. Of the 37 patients with erythema migrans, 12 had localized erythema migrans with no clinical evidence of systemic infection and 25 had evidence of early disseminated infection with either multiple erythema migrans lesions or systemic signs of infection manifested by constitutional symptoms, fever, meningitis, hepatitis, or carditis. Of the patients with localized erythema migrans, 11 of 12 were immunoreactive to the 41-kDa flagellar protein and 5 of 12 were immunoreactive to the 93-kDa protein (Table 2). Of the 25 patients with early disseminated disease, 19 of 25 reacted to the 93-kDa protein and 17 reacted to the 41-kDa antigen (Table 2). The 25 serum samples from patients with chronic disease were variably reactive with the 73-, 66-, 60-, 41-, 39-, 31-, 29-, and 22-kDa antigens. Twenty-two of 25 serum specimens recognized the 41-kDa protein, and all 25 recognized the 93-kDa protein (Table 2). Representative immunoblots from all three groups of patients studied are shown in Fig. 1. We conclude that in the populations we studied, these two proteins, the 41- and 93-kDa proteins, are frequently recognized antigens in all clinical stages of Lyme borreliosis. By contrast, samples from one of seven healthy controls, two of five rheumatoid arthritis patients, and four of seven patients with systemic lupus erythematosus reacted to the 41-kDa protein and no samples reacted to the 93-kDa antigen (data not shown).

TABLE 2. Antibody profiles of 62 patients with Lyme borreliosis[a]

Clinical manifestation	No. of patients	No. (%) of patients reactive to antigen	
		p41	p93
Erythema migrans	37	28 (75.7)	24 (64.9)
Localized rash	12	11 (91.7)	5 (41.7)
Early dissemination	25	17 (68.0)	19 (76.0)
Chronic disease	25	22 (88.0)	25 (100.0)

[a] Number of patients with an antibody response to the p41 and p93 antigens as measured by immunoblot analysis.

Identification and characterization of MAb to the 93-kDa protein of *B. burgdorferi*. To obtain specific reagents useful for the detection of the 93-kDa antigen, a murine MAb was developed. One hybridoma of 52 with anti-*B. burgdorferi* reactivity was found to have high specific activity against the 93-kDa protein. The hybridoma (MAb 181.1) producing this antibody was cloned and subcloned by limiting dilution, and the antibody produced was isotyped as immunoglobulin G1. The proteins of four strains of *B. burgdorferi* were separated by SDS-PAGE and immunoblotted (Fig. 2). As can be seen, MAb 181.1 recognized the 93-kDa protein in all strains. There was slight reactivity to a 32-kDa protein, which most probably represents reactivity to a degradation product of the 93-kDa protein. Similar to the previously described anti-93-kDa human MAb D4 (76), MAb 181.1 recognized the 93-kDa protein of *B. burgdorferi* with an apparent pI of 6.8 on an immunoblot of a two-dimensional gel (data not shown). By contrast, the MAb failed to bind to any protein when *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. coriaceae*, *T. denticola*, or *T. pallidum* was used as the antigen (data not shown).

Subcellular localization of p93. To localize the p93 antigen, *B. burgdorferi* was treated with Sarkosyl to generate soluble and insoluble fractions as previously described (20, 76). In our electron microscopic studies, the insoluble fraction contained components of both the protoplasmic cylinder and the flagella. However, it was not possible to determine whether our extraction procedure affected the integrity of the cylinder structure. Previously (76), we demonstrated by SDS-PAGE of the fractions that p93 partitioned with the Sarkosyl-insoluble fraction, whereas OspA and OspB, the two major outer surface proteins, partitioned with the Sarkosyl-soluble fraction. MAb 181.1 reacted to the 93-kDa

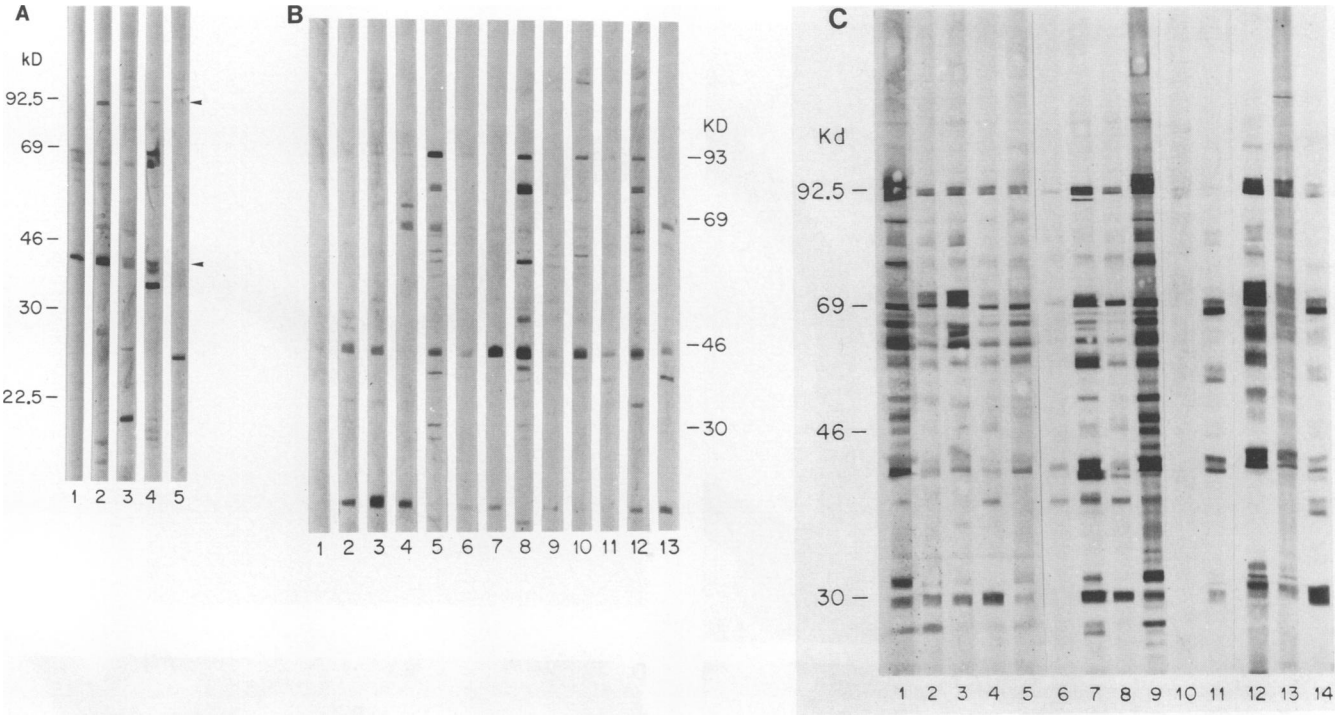

FIG. 1. Immunodetection of *B. burgdorferi* antigens by sera of patients with Lyme borreliosis. The antigens were separated by SDS-PAGE and then electrophoretically transferred to PVDF paper. The blots were reacted with human sera from 5 patients with localized erythema migrans (A), 13 patients with disseminated erythema migrans (B), and 14 patients with late Lyme borreliosis (C). Molecular mass standards (in kilodaltons) are shown on the left. The arrowheads refer to antigens recognized by MAbs specific for p93 and the 41-kDa flagella.

antigen in the whole organism and in the Sarkosyl-insoluble preparation. In an effort to further define the subcellular location of p93, we performed immune electron microscopy. The B31 strain was incubated with hybridoma supernatants containing either MAb 181.1 (anti-93-kDa antibody) or MAb 184.1 (anti-OspA antibody). Goat anti-mouse antibody complexed with colloidal gold was then used as a secondary antibody. Spirochetes fixed in gluteraldehyde showed a homogeneous distribution of gold particles when MAb 184.1 was used, whereas there was no binding of MAb 181.1 (Fig. 3A and B, respectively). MAb 181.1 failed to bind when the experiment was performed with unfixed spirochetes, whereas MAb 184.1 did bind. However, when Tween 20 was added to the blocking buffer, MAb 181.1 was noted to bind to the whole organism. We concluded that Tween 20 either caused the outer membrane to become more permeable or partially solubilized the membrane and uncovered the antigenic sites recognized by MAb 181.1. We therefore removed the outer envelope with Sarkosyl. The remaining fraction, which contained the insoluble portion of the protoplasmic cylinder and flagella, was reacted with the MAbs against OspA and p93 (Fig. 3C and D, respectively). In these experiments, MAb 184.1 failed to bind specifically to the protoplasmic cylinder (Fig. 3C), whereas MAb 181.1 bound to discrete sites along the protoplasmic cylinder (Fig. 3D). The 93-kDa antigen was distributed in a corkscrew distribution along the long axis of the protoplasmic cylinder in some micrographs. Multiple experiments confirmed these findings. These results along with our demonstration that p93 was not found in the Sarkosyl-soluble fraction indicate that this antigen is located on the protoplasmic cylinder of the organism.

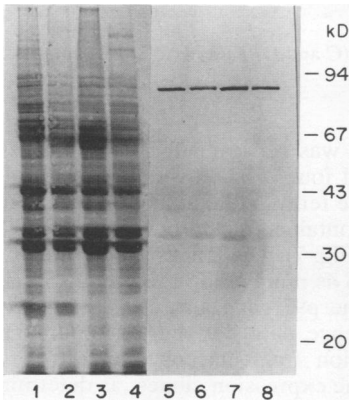

FIG. 2. Immunodetection of the 93-kDa antigen of four strains (B31, lane 1; 297, lane 2; Arc, lane 3; IP-2, lane 4) of *B. burgdorferi* by MAb 181.1. The antigens were separated by SDS-PAGE and then electrophoretically transferred to PVDF paper. Efficiency of transfer for each strain was demonstrated with amido black stain (lanes 1 to 4), and their reactivity to MAb 181.1 is shown in lanes 5 to 8, respectively. Molecular mass standards (in kilodaltons) are shown on the right.

Amino-terminal sequence and amino acid analysis. Protein separated by two-dimensional gel electrophoresis and electrophoretically transferred to PVDF paper (Fig. 4) was subjected to amino-terminal sequencing. The amino terminus contained the following hydrophilic sequence: ?EVDREKLKDFVNMDLEFVN (where "?" designates an undetermined residue). This sequence was confirmed by DNA sequence analysis of the genomic clone of p93. In the

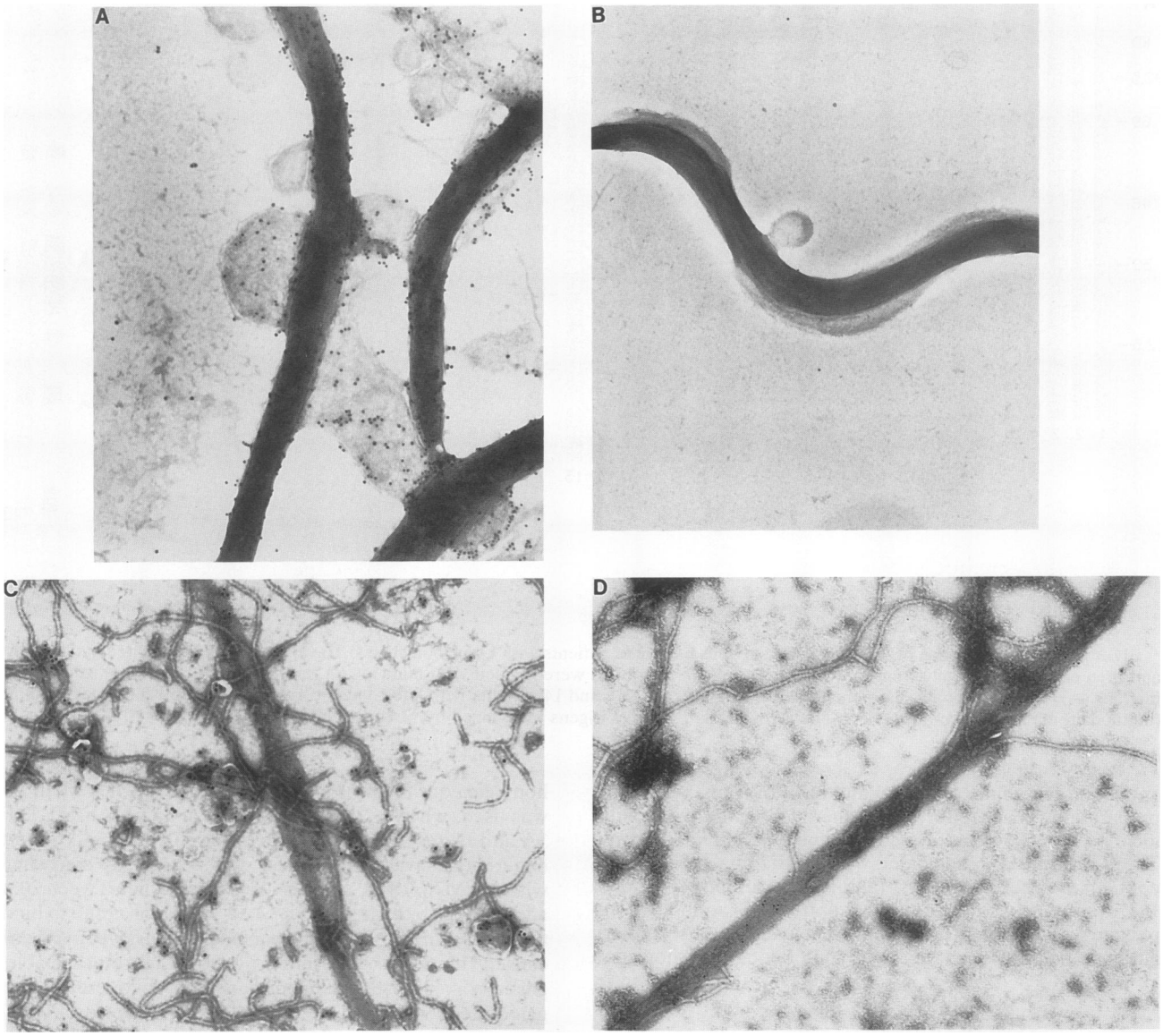

FIG. 3. Labeling of whole *B. burgdorferi* cells (A and B) and partially disrupted cells (C and D) with MAb 181.1 (anti-93-kDa protein) (B and D) and MAb 184.1 (anti-OspA) (A and C).

genomic clone, it is preceded by a hydrophobic 23-amino-acid polypeptide (see below).

Sequence analysis of the p93 gene. We constructed a λgt11 expression library of *B. burgdorferi* B31 genomic DNA and screened it with MAb 181.1. Four independent recombinant clones that reacted to MAb 181.1 were identified. We prepared lysogens from the immunoreactive phages and assessed immunoreactivity by immunoblotting the lysates. Murine MAb 181.1 recognized the *B. burgdorferi*–β-galactosidase fusion proteins from lysogen extracts and were not reactive to the *Escherichia coli* controls (Fig. 5). In addition to the major immunoreactive bands, there are several lower-molecular-weight bands that possibly represent products of internal initiation in the lambda clones. In addition, sera from 10 of 10 patients with Lyme borreliosis who demonstrated reactivity to the 93-kDa antigen of *B. burgdorferi* by immunoblot analysis reacted to the lysogen or immunoblots, while sera from none of 5 healthy controls reacted.

Insert DNA was retrieved from the clones by PCR and sequenced. All four clones overlapped and included a collinear sequence terminating in a stop codon, indicating that these clones contained the carboxy terminus of the putative p93 gene (see Fig. 7). The longest of these clones, D6, began at position 586 as numbered in reference 59. The remaining 5′ region of the p93 gene was cloned via inverse PCR by utilizing sequence data derived from our λgt11 clones. A *Bst*YI restriction fragment, encompassing sequences upstream from the expression clones, as determined by Southern blotting, was circularized, amplified by PCR with oligonucleotides 1 and 2 (Table 1), cloned, and sequenced. A second round of PCR was performed with a *Hin*dIII digestion to obtain the remainder of the upstream sequence. The resulting composite sequence revealed the complete genomic sequence for p93. The initiating ATG is preceded by consensus TATA and Shine-Dalgarno sequences (61). While this work was in preparation, Perng et al. (59) published an

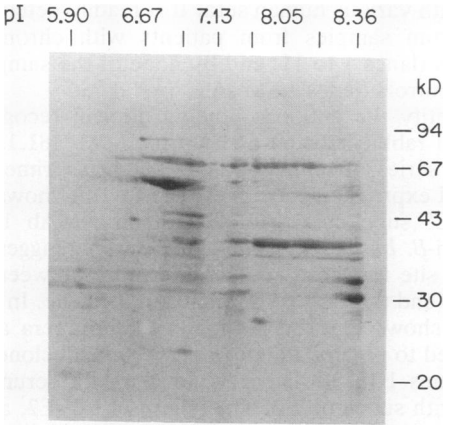

FIG. 4. Isolation of 93-kDa protein for amino-terminal sequence and analysis. Proteins of *B. burgdorferi* were separated by two-dimensional gel electrophoresis and electrophoretically transferred to PVDF paper. The 93-kDa protein was then excised. The molecular standards (in kilodaltons) are indicated on the right, and the pI value is listed above each lane.

almost identical sequence of a protein with an M_r of 83,000. The nucleotide sequence differed from that of Perng et al. at three positions (numbered as in reference 59), with changes as follows: 1446 (T→C), 1704 (C→G), and 2091 (G→A). The predicted amino acid sequence of these two isolates differed in only one position, Ser-568→Arg-568.

The amino-terminal sequence determined by microsequencing of the p93 protein was found to begin following a 23-amino-acid hydrophobic peptide in the derived amino acid sequence. This sequence most likely represents a cleaved signal peptide (57). The junction between these sequences, S-A-R, is consistent with its being a signal peptidase I site (80). Because cysteine is unstable under acid hydrolysis conditions, we oxidized cysteine to cysteic acid with performic acid and then analyzed for cysteic acid by using the Waters Pico Tag system. Cysteic acid was not detected under these conditions, nor were any cysteines

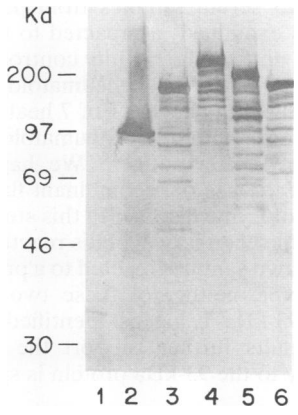

FIG. 5. Immunodetection of the 93-kDa lysogen by MAb 181.1. *E. coli* Y1089 lysate (lane 1), *B. burgdorferi* (B31) (lane 2), clone F10 (lane 3), clone D6 (lane 4), clone F2 (lane 5), and clone A5 (lane 6) were separated by one-dimensional SDS-PAGE and then electrophoretically transferred to PVDF paper and reacted with MAb 181.1. Molecular mass standards (in kilodaltons) are shown on the left.

TABLE 3. Amino acid composition of 93-kDa antigen measured by acid hydrolysis[a]

Amino acid	Amt in pmol (%)	Derived amino acid sequence composition (%)
His	43.36 (1.1)	0.43
Asp	377.38 (9.6)	9.71
Asn		6.43
Glu	567.29 (14.5)	9.00
Gly		4.29
Ser	435.40 (11.1)	9.29
Arg	215.58 (5.5)	3.00
Thr	224.67 (5.7)	3.71
Ala	278.79 (7.1)	3.86
Pro	165.71 (4.2)	2.29
Tyr	128.24 (3.3)	2.71
Val	242.82 (6.2)	5.86
Met	62.99 (1.6)	1.29
Ile	277.61 (7.1)	7.57
Leu	433.39 (11.1)	10.14
Phe	136.23 (3.5)	3.57
Lys	325.50 (8.3)	13.00
Cys[b]	0 (0)	0
Total	3,914.96	

[a] Aspartic and glutamic acids may be present in the amide forms as asparagine and glutamine. The determination of glycine was erratic and unreliable because of its presence in the electrophoresis buffers.

[b] Tryptophan and cysteine were not determined by acid hydrolysis. Cysteine composition was determined following performic acid hydrolysis and determination of cysteic acid.

encoded by the genomic clone of p93. Table 3 shows a comparison of the derived and chemically determined amino acid compositions of p93.

A fragment of the p93 gene encompassing nucleotides 586 to 2103 was labeled by random priming and used to probe Southern and Northern blots of the B31 strain of *B. burgdorferi* DNA and RNA, respectively. The p93 gene was found by pulsed-field gel electrophoresis to reside on chromosomal DNA (Fig. 6A and B). Southern blotting showed that this sequence is contained on a single restriction fragment (Fig. 6C) and encodes an mRNA of approximately 2.4 kb (Fig. 6D).

Mapping antigenic domains of p93. We demonstrated that the induced fusion proteins from the lysogens of the four putative p93 clones were recognized by both mouse and human MAbs and the sera of 10 patients with late Lyme borreliosis. We wished to identify the common portion of the phages in more detail. We therefore generated a series of recombinant constructs that expressed truncated portions of the gene in an *E. coli* expression plasmid, pATH3, which produces TrpE fusion proteins. We also cloned the p93 gene lacking the amino-terminal 20 amino acids into pET7HIS.2 (73) to generate p93Δ20, a nonfusion protein. The p93 λ clone D6 was used to generate fragments of the gene expressed as TrpE fusions in the pATH3 (39) vector. These constructs are diagrammed in Fig. 7 and are designated E1 through E5. We then used these constructs to map a region of the p93 molecule that is important for antibody recognition by rabbit polyclonal serum, mouse MAb 181.1, and a human MAb directed against p93.

Figure 8 shows the immunoreactivity of the p93Δ20 clone with 18 serum samples from patients with chronic Lyme borreliosis. The induced protein from clone p93Δ20 was

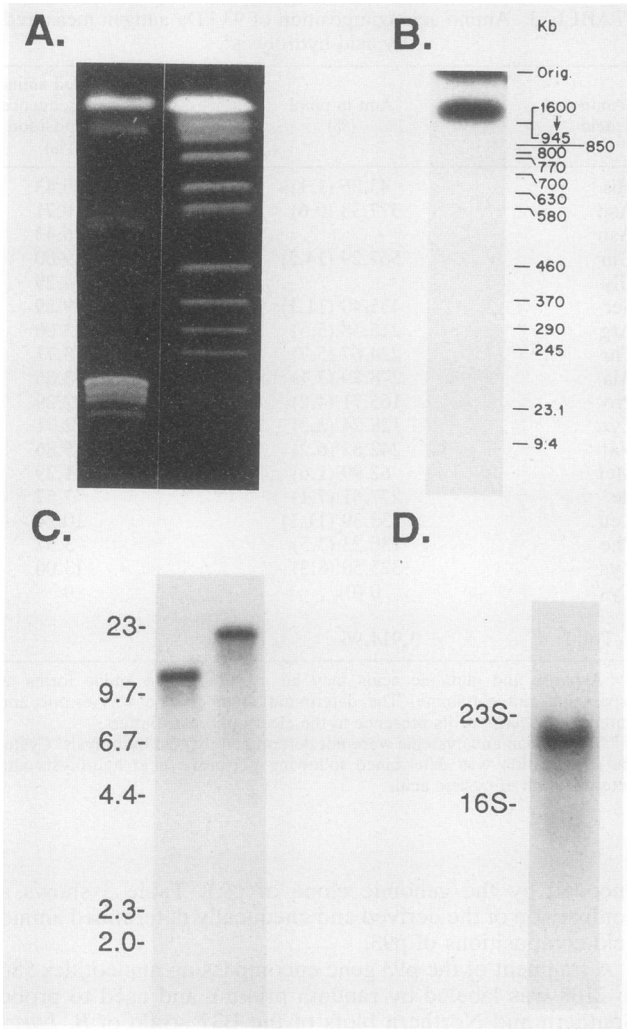

FIG. 6. Blot hybridization of p93 gene. (A) Ethidium bromide stain of pulsed-field gel electrophoresis of B31 strain DNA. Yeast chromosomal molecular weight markers were run in the right-hand lane. (B) Blot hybridization of the B31 DNA; (C) Southern blot of *Bgl*II- and *Eco*RI-digested B31 strain DNA; (D) Northern blot of RNA from the B31 strain. The positions of migration of 23S and 16S ribosomal RNA are indicated. In all cases, the probe was a fragment of the p93 gene (nucleotides 586 to 2103 in Fig. 7).

electrophoresed, blotted, and probed with these serum samples. A prominent band migrating at 93 kDa (arrowhead, Fig. 8) is seen in the amido black-stained blot of the induced clone (Fig. 8, lane 1). This band was excised, and the amino terminus was sequenced to determine that this band is p93Δ20. It was strongly recognized in 14 cases; however, four of the cases demonstrated weak reactivity. To define subregions of the protein important for antibody binding, we performed immunoblots with the expression subclones E1 through E5 (Fig. 9A), with a rabbit polyclonal serum directed against the whole *B. burgdorferi* organism. In addition to a number of nonspecific background bands that are seen in the vector control lane (lane 1), prominent bands are detected with the E4 clone (lane 5) and less prominent reactivity is noted with the E2 (lane 3) and E5 (lane 6) clones. No reactivity is seen with clones E1 and E3. Figure 9B shows the results of an immunoblot of the E4 subclone probed with various human sera. It is readily detected by 10 of 11 serum samples from patients with chronic Lyme borreliosis (lanes 1 to 11) and by none of the samples from normal controls (lanes 12 to 16).

To identify the antibody-binding domain recognized by polyclonal rabbit antisera and murine MAb 181.1, we performed a series of immunoprecipitation experiments using the pATH expression subclones. Figure 10A shows that the E4 and E5 subclones reacted with both MAb 181.1 and rabbit anti-*B. burgdorferi* polyclonal serum, suggesting that a binding site for these antibodies resides between nucleotides 1420 and the carboxy terminus of the gene. In contrast, Fig. 10B shows that the preimmune rabbit sera and MAb 181.1 failed to precipitate a complex from subclone E1, E2, or E3 (lanes 1 to 3). The polyclonal rabbit serum weakly reacted with subclone E2. The failure of E1, E2, and E3 to be immunoprecipitated by MAb 181.1 and the very limited reactivity of the polyclonal rabbit sera to only E2 suggest that the crucial structure for antibody recognition may comprise a complex epitope of noncontiguous residues within the carboxy-terminal region of the protein. In a similar manner, the human anti-p93 MAb, MAb D7, recognized the E4 fusion but failed to recognize E1, E2, or E3 (data not shown).

DISCUSSION

We have identified and partially characterized the 93-kDa immunodominant antigen of *B. burgdorferi*. In previous studies, we used two-dimensional gel electrophoresis analysis to define the immunodominant proteins of *B. burgdorferi* (46). During those studies, we noted that although the 93-kDa antigen was strongly immunogenic, it was remarkably sensitive to proteolysis even in the presence of proteolytic enzyme inhibitors. To identify and isolate this protein, it was necessary to avoid sonication and immediately solubilize the whole organism. Although this antigen is relatively sparse in the organism, it elicits a disproportionately strong immune response. Among the 37 patients with erythema migrans who had elevated anti-*B. burgdorferi* antibody titers recognized by ELISA and immunoblot analyses, both the 93- and 41-kDa proteins were recognized in more than 66% of patients, and all recognized either the 93- or 41-kDa protein. Of the 25 serum samples from patients with late Lyme borreliosis examined, all reacted to the 93-kDa antigen. By contrast, none of the healthy controls nor those with systemic lupus erythematosis or rheumatoid arthritis reacted to the 93-kDa antigen; however, 1 of 7 healthy controls and 50% (6 of 12) of those with other rheumatological conditions reacted with the 41-kDa antigen. We have conducted a separate study of the use of recombinant flagellum proteins for the diagnosis of Lyme disease. In this study, sera from 20 of 26 patients with secondary syphilis reacted to the 41-kDa protein, but only two samples reacted to a protein with an M_r of 93,000; however, neither of these two serum samples reacted to the 93-kDa lysogens identified in the present study. These results further support the conclusion that immunoreactivity to the 93-kDa protein is specific for Lyme borreliosis.

A MAb directed against the 93-kDa protein was reactive to all four strains of *B. burgdorferi* tested but failed to recognize an antigen on *T. denticola* or *T. pallidum* (data not shown). Furthermore, our MAb failed to bind to four other species of *Borrelia*: *B. hermsii*, *B. parkeri*, *B. turicatae*, and *B. coriaceae*. In contrast, we recently described a human MAb that reacted to the 93-kDa protein and cross-reacted to

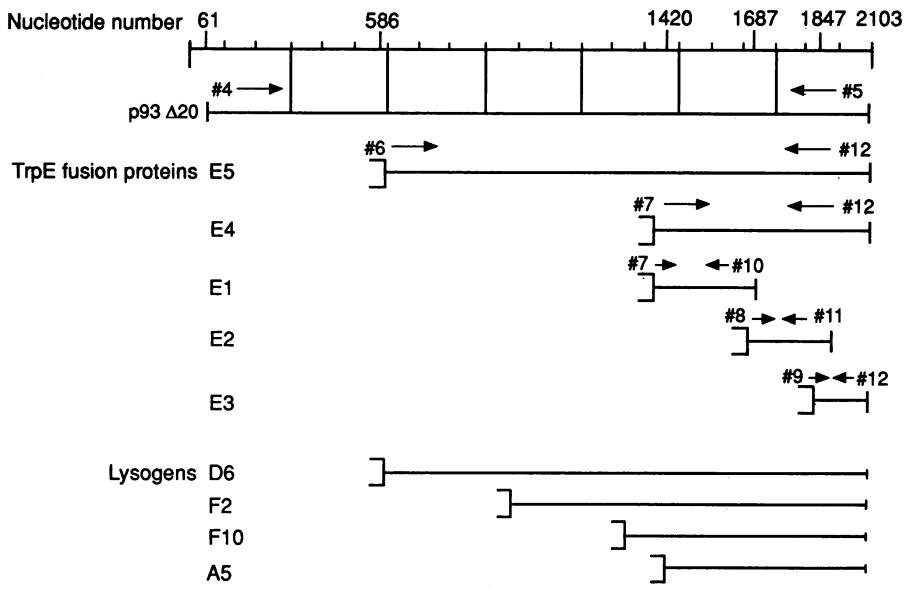

FIG. 7. Recombinant constructs of the p93 gene. The top line shows the nucleic acid sequence coordinates of the p93 gene as depicted in Fig. 8. p93Δ20 is a pET construct which deletes the amino-terminal 20 nucleotides of p93 expressed in the T7 vector pET7HIS.2. TrpE fusion proteins were constructed in the pATH3 vector and contain the indicated segments of p93. Lysogens indicate lambda gt11 lysogens isolated from the expression library. Solid lines indicate p93 sequences contained in the lambda arms. The location of primer nucleotides listed in Table 1 are indicated by arrows.

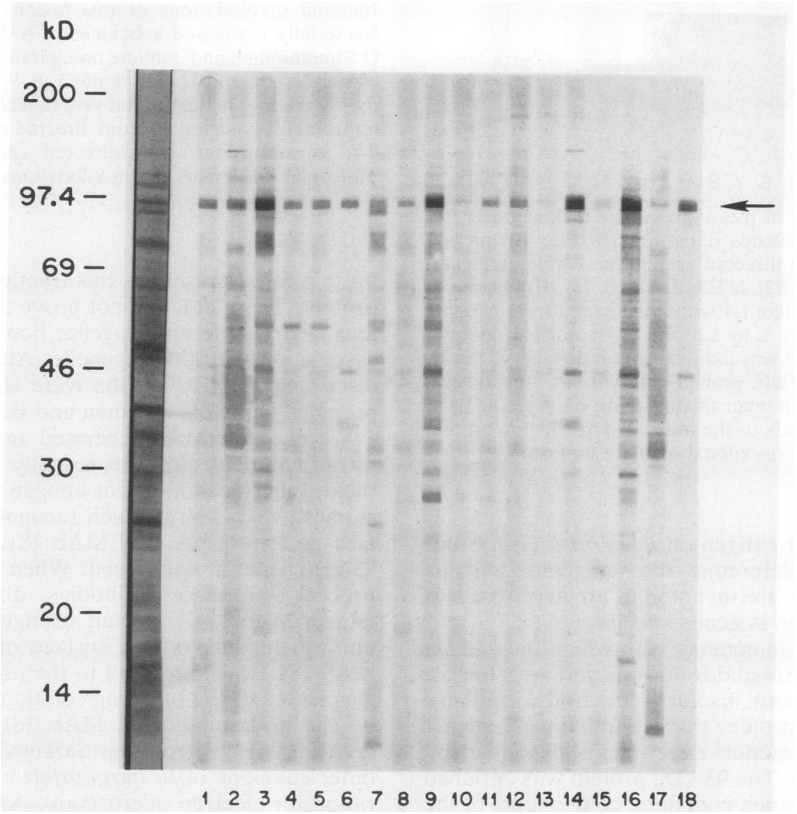

FIG. 8. Immunoreactivity of human sera against recombinant p93. The p93Δ20 clone was induced, lysed, electrophoresed, and blotted, and the resultant blot was probed with 18 serum samples from patients with Lyme borreliosis. The first lane contains an amido black-stained lysate of p93Δ20. Molecular mass markers (in kilodaltons) are indicated on the left.

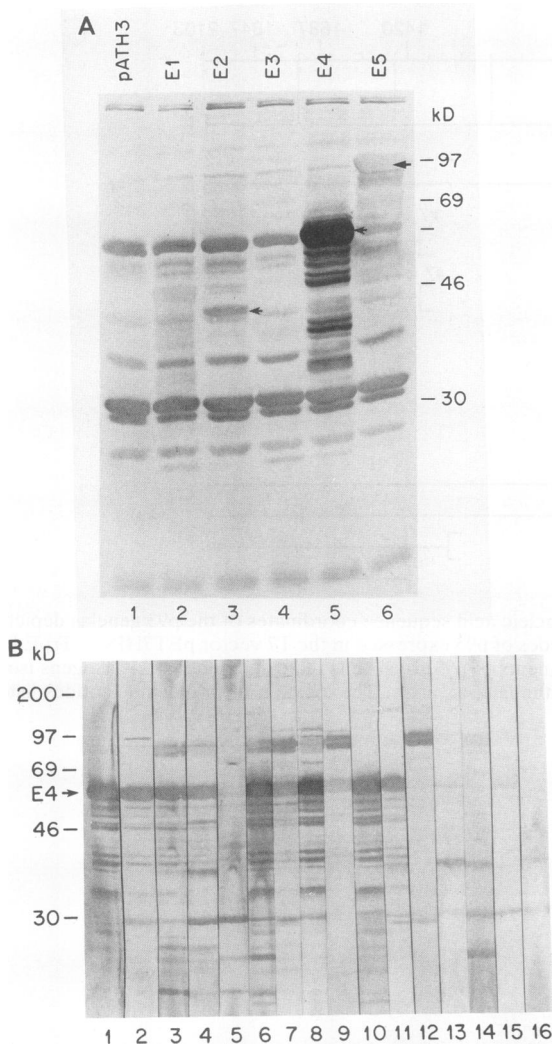

FIG. 9. Immunoreactivity of p93 expression subclones. (A) Immunoblot of expression subclones (illustrated in Fig. 10) probed with rabbit polyclonal serum directed against the whole organism. Lanes: 1, pATH3; 2, E1; 3, E2; 4, E3; 5, E4; 6, E5. (B) Immunoreactivity of TrpE fusion protein E4 with human sera from patients with Lyme borreliosis (lanes 1 to 12) and from normal controls (lanes 13 to 17). The E4 clone was induced with indole acrylic acid, transferred to Immobilon-PVDF, probed with various human sera, and developed with goat anti-human alkaline phosphatase. The band labeled E4 (arrow) corresponds to the induced TrpE fusion protein E4. Molecular mass markers (in kilodaltons) are indicated.

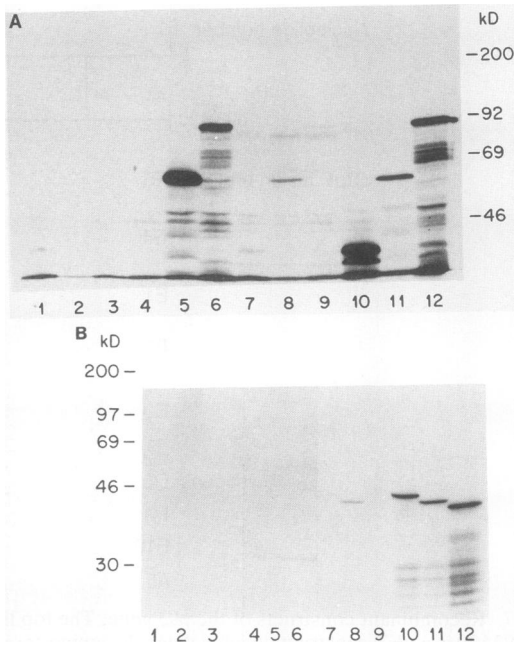

FIG. 10. (A) Immune precipitation of TrpE fusion proteins E4 and E5. Bacterially expressed proteins were labeled in vivo with [^{35}S]methionine, and the cells were lysed and immunoprecipitated. Lanes: 1, 4, 7, and 10, pATH3; 2, 5, 8, and 11, E4 clone; 3, 6, 9, and 12, E5 clone. Immune precipitations were done with preimmune rabbit serum (lanes 1 to 3), polyclonal rabbit serum directed against the whole *B. burgdorferi* organism (lanes 4 to 6), MAb 181.1 (lanes 7 to 9), and rabbit serum directed against TrpE (lanes 10 to 12). (B) Immune precipitations of p93 fusion proteins E1, E2, and E3. Bacterially expressed subclones of p93 were labeled in vivo with [^{35}S]methionine and immune precipitated. Lanes: 1, 4, 7, and 10, E1 clone; 2, 5, 8, and 11, E2 clone; 3, 6, 9, and 12, E3 clone. Antisera used were preimmune rabbit sera (lanes 1 to 3), MAb 181.1 (lanes 4 to 6), rabbit polyclonal serum directed against *B. burgdorferi* (lanes 7 to 9), and rabbit serum directed against TrpE (lanes 10 to 12). Molecular mass markers (in kilodaltons) are indicated.

a higher-molecular-weight antigen on *B. hermsii* (76). Studies are in progress to determine the antigenic domains specific for *B. burgdorferi*. Further studies are in progress to define whether this antigen is genus specific.

To define the cellular components with which the 93-kDa protein is associated, we treated *B. burgdorferi* with Sarkosyl to generate soluble and insoluble fractions. In these experiments, the protein profiles that we found were almost identical to the SDS extraction experiments described by Coleman and Benach (20). The 93-kDa protein was enriched in the insoluble fraction which contained components of the protoplasmic cylinder and the flagella of the organism. Furthermore, examination of the SDS-PAGE profile of the cesium chloride-purified endoflagella showed the 93-kDa protein to be present in this fraction along with the 41-kDa protein. These data did not prove that the 93-kDa antigen is localized on the endoflagella; however, they suggested an association with this organelle. Although not noted in their discussion, similar results were shown in the figures of a previous study by Coleman and Benach (20).

We used the MAb generated against this antigen to perform immunoelectron microscopy to define more precisely the cellular location of this antigen in the organism. In these studies, MAb 184.1, which recognizes the major outer surface protein OspA, and MAb 181.1, which recognizes the 93-kDa protein, were used. When the whole organism was probed with these antibodies, different results were obtained. MAb 184.1 reacted strongly to *B. burgdorferi*, with uniform binding on the surface of the organism, whereas MAb 181.1 failed to bind to the organism. When Tween 20 was added to the blocking solution to decrease background on the grid, binding of MAb 181.1 to *B. burgdorferi* increased. We inferred that Sarkosyl treatment disrupted the outer envelope of *B. burgdorferi* and have used this preparation for electron microscopy. As can be seen in Fig. 5D, MAb 181.1 obviously reacted to the protoplasmic cylinder, whereas MAb 184.1 failed to do so. From these and our previous studies (76), it is apparent that Sarkosyl treatment

effectively solubilizes the outer membrane, as evidenced by the removal of the abundant major outer surface proteins leaving the flagella and at least a remnant of the protoplasmic cylinder. It is not possible to determine whether p93 is located on the surface of the protoplasmic cylinder; however, given its location and its predicted alpha helical structure, it is tempting to speculate that the 93-kDa protein may be a constituent of a helical fibril on the surface of the protoplasmic cylinder. This would be analogous to the structure described for *Spirochaeta stenostrepta* (31). In this regard, it is of interest that the amino acid composition of the helical fibrils of *S. stenostrepta* is similar to that which we found in the 93-kDa protein, with relatively high content of aspartic acid, glutamic acid, alanine, glycine, and leucine. It appears that the two immunodominant antigens, the 41- and 93-kDa proteins, localize to the flagella and protoplasmic cylinder, respectively. It remains unclear how these two dominant antigens become accessible for antigenic recognition, whereas the more plentiful and apparently more accessible outer surface proteins, OspA and OspB, do not.

The gene encoding the 93-kDa protein was identified in four recombinant clones of a lambda gt11 genomic expression library. Lysogens of these clones were recognized by MAb 181.1 and by sera from 10 of 10 patients with late Lyme borreliosis but not by sera from healthy controls or from patients with secondary syphilis (data not shown). Southern blotting of a pulsed-field gel of *B. burgdorferi* genome demonstrated that the genes encoding both the 93- and 41-kDa proteins (data not shown) are located on the linear chromosome. By contrast, the genes encoding the major outer surface proteins, OspA and OspB, in which the immune response is limited, are encoded by the 49-kb linear plasmid (14, 33).

While this work was in preparation (45), Perng et al. (59) reported the nucleotide sequence that we have identified in the present work. The sequence reported by these workers corresponds to an 83-kDa protein but is nearly identical to our sequence. The DNA sequence of the p93 gene shows that the protein begins with a hydrophobic leader sequence followed by the peptide S-A-R, which conforms to the cleavage site for *E. coli* signal peptidase I (SPaseI) (79). Interestingly, our N-terminal peptide sequencing of isolated 93-kDa protein began following this site, suggesting that the protein is processed through an SPaseI-like pathway. Analysis of the derived amino acid sequence of the 93-kDa protein (58) revealed a highly hydrophilic domain predicted to be alpha helical over a domain of 300 amino acids in the middle portion of the sequence. This region is significantly homologous to the alpha helical filament portion of the myosin heavy chain of *Dictyostelium discordium* showing 16% amino acid identity. The observed homology to the filament domain of myosin may indicate that the 93-kDa protein plays a structural role.

We have identified the 93-kDa protein to be a dominant antigen in patients acutely and chronically infected with *B. burgdorferi*. Further studies with other European and Asian strains are needed to determine whether there is polymorphism of this antigen. We have expressed the 93-kDa protein as well as its immunodominant domains in pATH expression systems. This protein may be particularly useful in combination with other recently identified antigens or other *B. burgdorferi*-specific components which have been cloned (59, 65) for the development of sensitive and specific serodiagnostic tests. The availability of these reagents will be useful for the further definition of the role that this protein may play in the bacterial function and pathogenesis of Lyme disease. Expression and production of this antigen and its components may prove to be useful for the systematic assessment of the utility of this protein as a diagnostic reagent for the early diagnosis of Lyme borreliosis.

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