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Conformational Nature of the *Borrelia burgdorferi* Decorin Binding Protein A Epitopes That Elicit Protective Antibodies

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Decorin binding protein A (DbpA) has been shown by several laboratories to be a protective antigen for the prevention of experimental *Borrelia burgdorferi* infection in the mouse model of Lyme borreliosis. However, different recombinant forms of the antigen having either lipidated amino termini, approximating the natural secretion and posttranslational processing, or nonprocessed cytosolic forms have elicited disparate levels of protection in the mouse model. We have now used the unique functional properties of this molecule to investigate the structural requirements needed to elicit a protective immune response. Genetic and physicochemical alterations to DbpA showed that the ability to bind to the ligand decorin is indicative of a potent immunogen but is not conclusive. By mutating the two carboxy-terminal nonconserved cysteines of DbpA from *B. burgdorferi* strain N40, we have determined that the stability afforded by the putative disulfide bond is essential for the generation of protective antibodies. This mutated protein was more sensitive to thermal denaturation and proteolysis, suggesting that it is in a less ordered state. Immunization with DbpA that was thermally denatured and functionally inactivated stimulated an immune response that was not protective and lacked bactericidal antibodies. Antibodies against conformationally altered forms of DbpA also failed to kill heterologous *B. garinii* and *B. afzelii* strains. Additionally, nonsecreted recombinant forms of $DbpA_{N40}$ were found to be inferior to secreted lipoprotein $DbpA_{N40}$ in terms of functional activity and antigenic potency. These data suggest that elicitation of a bactericidal and protective immune response to DbpA requires a properly folded conformation for the production of functional antibodies.

Lyme disease (41) or Lyme borreliosis, is caused by a group of related tick-borne spirochetes classified as *Borrelia burgdorferi* sensu lato (including *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*). Recent clinical trials have shown that monovalent recombinant subunit vaccines composed of the *Borrelia* outer surface protein A (OspA) lipoprotein were efficacious through two Lyme disease transmission seasons (40, 42). The mechanism of this protective effect differs from that of other vaccines. The OspA protein is expressed by spirochetes in the tick midgut, but this protein is down regulated during tick engorgement (13) and in the mammalian host (7). Protection by immunization with OspA therefore involves prevention of transmission of the spirochetes from the tick to the mammalian host and is dependent on having a critical threshold level of antibodies at the time of the tick bite (12). The addition of mammalian host stage antigens to the OspA vaccines may extend the duration or enhance the level of protective efficacy of such transmission-blocking vaccines (28). Alternatively, vaccines composed of one or more mammalian-stage antigens may be effective without OspA.

Several *B. burgdorferi* proteins expressed in the mammalian stage have been shown to be effective vaccines for preventing infection in laboratory animals challenged by experimental or natural routes. These protective antigens include OspC, P35/BBK32, P66/Oms66, and decorin binding protein (14, 17, 19, 20, 25, 27, 34). Decorin binding proteins A and B (DbpA and DbpB) are *B. burgdorferi* lipoproteins (23, 27) that are surface exposed and may act as spirochetal adhesins (24). We have demonstrated that immunization of mice with DbpA protected them from challenge with cultured spirochetes (27), and others (17, 25) have confirmed this protection. DbpA is expressed in vivo during spirochetemia in the mouse model (7) and is recognized by human Lyme disease patient sera (8, 29). These data suggest a potential role for DbpA in an improved Lyme vaccine.

Studies of DbpA vaccine effectiveness in other laboratories have relied on *Escherichia coli* vectors expressing cytosolic products as fusions to affinity tag sequences, a commonly used strategy for generating recombinant immunogens. However, recombinant cytosolic DbpA expressed as amino-terminal fusions to either polyhistidine (25) or glutathione *S*-transferase (GST) (17) was less than completely effective in these other studies. In the present study we sought to determine which form of the DbpA antigen would be most effective as a vaccine antigen and whether the protective efficacy of the protein was conformationally dependent. Due to the ability to measure ligand binding activity of the DbpA forms, we took advantage of the opportunity of being able to correlate function and therefore correct folding with the ability to elicit a protective response.

MATERIALS AND METHODS

***B. burgdorferi* and culture conditions.** Cloned strains of *B. burgdorferi* sensu stricto isolate N40 (3) and mouse-adapted *B. afzelii* isolate PKo (2) were donated by S. Barthold. Isolate *B. garinii* VSBP was donated by R. Johnson (44). Spirochetes were propagated in tightly closed containers at 33 or 37°C in modified Barbour-Stoenner-Kelly (BSKII) medium (1). The cell densities of these cultures were determined by dark-field microscopy at ×400.

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Expression and purification of recombinant proteins. Expression in *E. coli* BL21(DE3)pLysS and purification of chimeric lipoprotein Lpp:DbpA$_{N40}$ have been described previously (7). Lpp:DbpA$_{N40}$H$_6$ was expressed from plasmid pWCR129 (27) in *E. coli* BL21(DE3)pLysS. Membrane-associated proteins were solubilized in the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as described previously (7), and LppDbpA$_{N40}$H$_6$ was purified with minor modifications as follows. The CHAPS soluble fraction was loaded onto Ni-nitrilotriacetic acid Sepharose (Qiagen, Valencia, Calif.) equilibrated in 20 mM $NaPO_4$ (Na_2HPO_4 and NaH_2PO_4 mixed to attain pH 8.0)–300 mM NaCl–10 mM CHAPS. The protein was eluted using a linear gradient of the starting buffer with 250 mM imidazole. The eluted protein was dialyzed against phosphate-buffered saline (PBS) (pH 7.4) (HyClone, Logan, Utah)–8 mM CHAPS

H$_6$-DbpA$_{N40}$ was expressed from plasmid pWCR135 in *E. coli* M15/pREP4. Plasmid pWCR135 was produced by insertion of a *Bam*HI-*Hin*dIII fragment from pWCR130 (7) encoding the mature DbpA$_{N40}$ into the expression plasmid pQE31 (Qiagen Inc., Valencia, Calif.). The cytosolic H$_{10}$-DbpA$_{N40}$ was expressed using a *Bam*HI-to-*Hin*dIII fragment encoding the mature DbpA$_{N40}$ from pWCR129 that was ligated into the newly constructed expression vector pWCR138 to yield pWCR139. Plasmid pWCR138 is a pET19b derivative made as follows: pET19b was cleaved with *Bam*HI and *Blp*I and was ligated to the *Bam*HI-*Blp*I fragment containing the multiple-cloning site of pET26b to generate a plasmid that can add a histidine tag to either the amino terminus or the carboxy terminus of the expressed product. BL21(DE3)pLysS/pWCR139 was grown, and expression of H$_{10}$-DbpA$_{N40}$ was induced as described previously (7). M15/pREP4/pWCR135 was grown in Luria-Bertani broth with carbenicillin at 50 μg/ml and kanamycin at 50 μg/ml and induced for 2 h with 1 mM isopropyl-β-D-thiogalactoside (IPTG). The cell pellets for both cytoplasmically expressed proteins were resuspended into 20 mM $NaPO_4$ (pH 8.0)–300 mM NaCl–1 mM benzamidine–0.2 mM phenylmethylsulfonyl fluoride, and the cells were disrupted by microfluidization at 10,000 lb/in^2. The cell debris was removed at 10,000 × *g* for 10 min, and membrane fractions were removed by centrifugation at 100,000 × *g* for 1 h. The soluble fraction was loaded onto a Ni-nitrilotriacetic acid column equilibrated in the resuspension buffer, and the histidine-tagged protein was eluted with a linear gradient up to 500 mM imidazole.

A plasmid encoding the mutant protein LppDbpA$_{N40}$H$_6$ (Cys 176, 191 Ser) was made from pWCR129 by introducing two single-nucleotide changes using QuickChange (Stratagene Cloning Systems, La Jolla, Calif.). For mutagenesis at the Cys-176 codon of the DbpA$_{N40}$ gene, the following oligonucleotides were used: 5′-CAAAAAACTACaGCGCCCTTGAAAAG-3′ and 5′-CTTTTCAA GGGCGCtGTAGTTTTTTG-3′. After the mutation at cysteine 176 was confirmed by sequencing (38), the singly mutated plasmid was used for a second round of mutagenesis to mutate the Cys-191. The following oligonucleotides were used to generate the double mutant: 5′- CTGATGAAAAAaGCAAAAA TAAC-3′ and 5′-GTTATTTTTGCtTTTTTCATCAG. The cysteine mutant protein was expressed and isolated as described above for the wild-type protein.

The gene encoding DbpA from *B. afzelii* PKo was identified by a PCR-based approach with primer pairs 10F4 plus WR25 and BM73 plus WR39 that we previously used for amplification of other *B. afzelii* alleles of *dbpA* (38). DNA encoding the mature DbpA$_{PKo}$ was amplified by PCR using the following oligonucleotides: 5′-CCGGATCCTAGTTTAACAGGAAAAGCT-3′ and 5′-CGAA GCTTAGTCGACTTTTTGATTTTTAGTTTG-3′. This PCR product was digested with *Bam*HI and *Hin*dIII, and the subsequent fragment was ligated into the same sites of the lipoprotein expression vector pT7Lpp2 (27) to yield plasmid pCMB01. Expression from pCMB01 and extraction of Lpp:DbpA$_{PKo}$ from the CHAPS-soluble fraction of BL21/DE3/pLysS/pCMB01 were carried out as previously described for other lipoprotein DbpAs (7). The CHAPS-soluble proteins were passed over a MacroPrep Q (Bio-Rad, Hercules, Calif.) column equilibrated in 20 mM $NaPO_4$–100 mM NaCl–10 mM CHAPS (pH 7.4). Lpp:DbpA$_{PKo}$ was found in the flowthrough fraction of the column and subsequently applied to a ceramic hydroxyapatite type 1 column (Bio-Rad). Lpp:DbpA$_{PKo}$ was eluted into 300 mM $NaPO_4$ (pH 7.4)–10 mM CHAPS.

DNA encoding the mature DbpA from *B. garinii* VSBP (38) was amplified by PCR using primers 5′-CCGGATCCCGGCTTAACAGGAGAAACTAA-3′ and 5′-CTGTCTAAGCTTAGTCGACTGTAGTAGTAGCAGTGT-3′. This PCR product was digested with *Bam*HI and *Sal*I, and the resulting fragment was ligated into the same sites of pT7Lpp2 to yield plasmid pWCR133 expressing Lpp:DbpA$_{VSBP}$H$_6$. Methods used for expression and purification of Lpp:DbpA$_{VSBP}$H$_6$ were similar to those described previously (7).

The purity of the recombinant proteins was >90% as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with Coomassie blue staining or Sypro Red (Molecular Probes, Eugene, Oreg.) staining

Decorin binding assays. A solid-phase plate assay was developed for measuring binding to decorin. Recombinant human decorin (39) was obtained from David Mann. Wells of Maxisorb (Nunc) plates were coated with 100 μl of decorin solution at 0.5 mg/ml in PBS and incubated overnight at 4°C. After unbound decorin was decanted, the plates were blocked with 5% nonfat dried milk in PBS–0.1% Tween 20. Samples of DbpA were applied to the plate in blocking buffer at different concentrations and incubated for 1 h at room temperature. The plates were washed with PBS-Tween and incubated with a 1:2,500 dilution of rabbit polyclonal sera against Lpp:DbpA$_{N40}$. Rabbit hyperimmune antiserum was raised as described previously (27). The plates were washed and incubated with a 1:2,500 dilution of goat anti-rabbit–horseradish peroxidase conjugate antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 1 h at room temperature. The binding of DbpA-antibody complex to the decorin-coated plate was visualized by addition of 2,2′-aminobis(3-ethylbenzthiazolinasulfonic acid) (ABTS) substrate (Kirkegaard & Perry Laboratories). For assays of the heat-denatured protein, the DbpA was incubated at 65°C for 1 h prior to assay. Urea denaturation was performed by addition of urea to 8 M and incubation of the sample for 30 min at 37°C. The renaturation was performed by dilution 1:100 into PBS–0.1% Tween 20 or by dialysis into PBS–10 mM CHAPS.

A Western blot format assay (24, 27) was also used to detect decorin binding activity when the homologous DbpA antiserum was not available. Decorin was conjugated with biotin using the biotin-XX-sulfosuccinimidyl ester reagent (Molecular Probes Inc.) as specified by the manufacturer, and biotinylated decorin was used to probe filters blotted with candidate DbpAs. Recombinant OspC$_{N40}$, a protein whose size and charge are similar to DbpA but which lacks decorin binding activity (23), was donated by B. Guo and M. Höök and was used here as a negative control. Decorin binding activity was detected with a streptavidin-alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories) using enhanced chemifluorescence reagent (Amersham-Pharmacia Biotech, Piscataway, N.J.) for visualization.

Immunization and challenge. Pathogen-free female C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and used at 6 to 8 weeks of age. Prior to immunization, DbpA or control antigens were formulated in one of five ways. Proteins were emulsified 1:1 (vol:vol) with Freund's adjuvant (Difco) or adsorbed with the aluminum adjuvant Alhydrogel (Superfos Biosector, Kvistgård, Denmark) and administered either intraperitoneally or subcutaneously in a volume of 100 μl. The primary immunizations with Freund's adjuvant were performed in complete Freund's adjuvant and were followed by a second immunization 4 weeks later in incomplete Freund's adjuvant. Some mice were immunized a third time after another 4-week interval. Protein was diluted in PBS and injected into vials containing trehalose dimycolate plus monophosphoryl lipid A, as supplied by the manufacturer (RIBI Immunochem Research, Inc., Hamilton, Mont.). The vials were vortexed until an emulsion was formed, and the material was injected into mice subcutaneously at a volume of 200 μl. Quil A (Sigma, St. Louis, Mo.) was added to protein to a final concentration of 250 μg/ml. For Alhydrogel + Quil A, protein was allowed to bind to Alhydrogel first and then Quil A was added to a final concentration of 250 μg/ml. Immunogen was injected subcutaneously at a volume of 100 μl. The mice were challenged at 2 weeks after the final immunization with 10^4 *B. burgdorferi* N40. Infection status was determined from BSKII cultures of five tissues as described previously (27).

In vitro growth inhibition assay and ELISA. Microwell titer determinations for determination of growth-inhibitory titers and immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) titers of DbpA antisera were performed as described previously (27). Spirochetes in BSKII growing for 3 to 5 days at 33°C, or 37°C for *B. afzelii* and *B. garinii*, in the presence of serially diluted antiserum were enumerated by dark-field microscopy at a magnification of ×400. The inhibition end-point titer was determined as the dilution of antiserum promoting a >85 to 90% reduction in the number of motile spirochetes compared to control samples, which typically yielded 50 to 75 spirochetes per microscopic field.

In vitro proteolysis. Samples of H$_{10}$-DbpA$_{N40}$, Lpp:DbpA$_{N40}$, Lpp:DbpA$_{N40}$-H$_6$, and the corresponding cysteine mutant form were incubated at either room temperature or 37°C in the presence of 10% (wt/wt) clostripain (Worthington Biochemical Corp., Lakewood, N.J.)–10 mM CHAPS–1 mM $CaCl_2$. Clostripain was activated in 2.5 mM dithiothreitol–1 mM $CaCl_2$ prior to use in the assay. The proteolysis was terminated at different times by addition of reaction aliquots into EDTA to a concentration of 25 mM. Samples were boiled in SDS sample buffer and run on a NuPage (Invitrogen, Carlsbad, Calif.) 10% acrylamide gel in morpholineethanesulfonic acid (MES) buffer provided by the vendor. Proteins were visualized on a Molecular Dynamics Storm (Amersham-Pharmacia Biotech, Piscataway, N.J.) imager by fluorescence staining with Sypro Red.

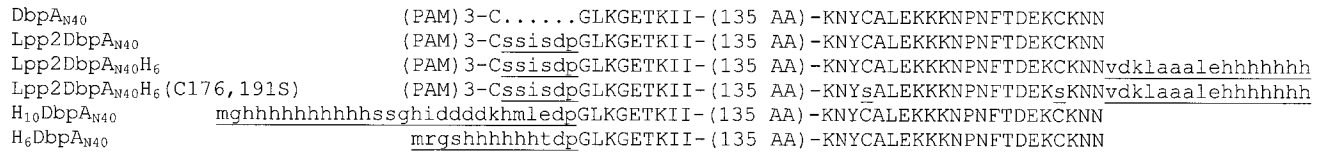

FIG. 1. Sequence comparison of five recombinant forms of DbpA$_{N40}$ and the natural sequence. The amino-terminal and carboxy-terminal residues of the mature lipoprotein forms of DbpA$_{N40}$, both natural (top line) and chimeric recombinant lipoprotein (Lpp) forms, and the nonlipoprotein forms (H$_{10}$ or H$_6$) are shown. (PAM)3- indicates the presumed tripalmitoyl posttranslational modification to the amino-terminal cysteine of the lipoprotein forms. Nonnatural vector-encoded or mutated residues are underlined and lowercase. The central 135 amino acids are omitted from the diagrams for clarity.

Circular dichroism (CD). All spectra were obtained using a Jasco 810 spectrapolarimeter (Jasco, Easton, Md.) equipped with a Peltier temperature controller in a 1-mm pathlength cell. Thermal denaturations were performed while collecting data at 220 nm using a scan rate of 1°C/min from 20 to 80°C. Samples for denaturation were used at a concentration of 0.1 mg/ml in PBS with 10% (wt/vol) CHAPS detergent. For full spectral analysis, samples were dialyzed into 20 mM $NaPO_4$–10 mM Zwittergent 3-12 (pH 8.0) prior to analysis to remove CHAPS interference of the spectra. Full-scan spectra were obtained by scanning from 260 to 180 nm at a rate of 100 nm/min and averaging two spectra.

RESULTS

Protection of mice from *Borrelia* infection by immunization with different recombinant forms of DbpA. We have shown previously that both passive and active immunization can protect mice from infection with both cultured (27) and in vivo-derived (7) borreliae. Our previous work suggested that different forms of the antigen may vary in their vaccine efficacy. Accordingly, we set out to determine the form of the antigen giving the most protective immune response. We expressed DbpA from *B. burgdorferi* strain N40 either as translocated/lipidated forms or as a cytoplasmically expressed form for the present study. The salient amino terminal and carboxy-terminal sequences of these different constructs are shown in Fig. 1.

Mice were immunized two or three times with various adjuvant formulations of three recombinant forms of DbpA$_{N40}$ differing in their expression compartment, posttranslational processing, and carboxy termini. Biological potencies, including in vitro borreliacidal activity and protective efficacy, of immune responses to these various vaccine regimens were compared for three representative experiments (Table 1). Secreted and acylated lipoprotein forms of DbpA (Lpp:DbpA$_{N40}$ and Lpp:DbpA$_{N40}$H$_6$) elicited humoral immune responses of higher biological activity than immune responses against nonsecreted DbpA (H$_6$DbpA$_{N40}$) even though serum IgG levels were comparable by ELISA. Nonsecreted H$_6$DbpA$_{N40}$ required three vaccinations to elicit borreliacidal antibodies and protection (compare experiments 1 and 2). Lpp:DbpA$_{N40}$ elicited measurable levels of growth-inhibiting antibodies after two vaccinations, and the growth-inhibiting activity increased further after three vaccinations.

Lpp:DbpA$_{N40}$H$_6$ with the carboxy-terminal extension was even more potent than Lpp:DbpA$_{N40}$ without this tag (experiments 2 and 3). This has also been observed with recombinant DbpA lipoproteins from *B. burgdorferi* strain B31, suggesting that the basis of the difference is conserved between these two isolates (data not shown). We have also determined that the lipidated histidine-tagged form of the protein is effective at eliciting a protective immune response in the absence of adjuvant (28). Antiserum against an unrelated *Haemophilus influenzae* antigen expressed similarly to a chimeric lipoprotein with the carboxy-terminal polyhistidine tag had negligible reactivity with *B. burgdorferi*, arguing against the trivial explanation that the polyhistidine sequence itself elicited biologically relevant antibodies (data not shown). Lpp:DbpA$_{N40}$H$_6$ was the superior antigen among these three recombinant forms of DbpA for each of the five adjuvant formulations tested. Interestingly, antiserum from mice immunized three times with the Alhydrogel formulation of Lpp:DbpA$_{N40}$H$_6$ had growth-inhibitory activity comparable to that of antiserum against other adjuvant formulations that elicited much higher total specific IgG levels measured by ELISA.

Overall, there was a strong correlation between the protective efficacies of the various DbpA formulations and the in vitro growth-inhibitory activity of their antisera but no correlation with the total DbpA-specific IgG elicited. When antisera from groups of mice showing partial protection were analyzed individually for growth-inhibitory activity, the mice that were vaccine failures had the lowest growth-inhibitory titers within their immunization groups (data not shown). Since all three recombinant forms of the antigen (Fig. 1) share the full mature sequence of DbpA, we then investigated a structural explanation for the differences in their potency.

Recombinant chimeric DbpAs differ in their functional activity. In vitro studies have shown that DbpA binds with high specificity to decorin, an extracellular matrix dermatan sulfate proteoglycan (23, 24). At this time, DbpA is one of the few *B. burgdorferi* surface proteins with a biochemically defined and quantifiable function. It seemed likely that this activity was dependent on proper DbpA conformation and, if so, we could potentially exploit this structure-function relationship as a tool for defining conformational serological epitopes. We developed a solid-phase equilibrium binding assay to assess the ability to discriminate among the different forms of DbpA in their binding to decorin. DbpA bound to decorin-coated microwell plates in a concentration-dependent and saturable manner. Decorin bound similarly to immobilized DbpA (data not shown). To minimize variables in plate-coating conditions among different DbpA forms, we used the immobilized decorin format. As shown in Fig. 2A, the secreted and lipidated forms of DbpA bind to the decorin on the plate while the cytoplasmically produced amino-terminal histidine-tagged DbpA does not bind substantially at the same concentration. For this comparison, we substituted H$_{10}$DbpA$_{N40}$, another cytosolic form of the protein, for H$_6$DbpA$_{N40}$ since they were essentially equivalent in decorin binding activity and CD spectra (data not shown). Additionally, H$_{10}$DbpA$_{N40}$ was expressed and purified in higher yield, and use of this form allowed confirmation that

TABLE 1. Comparison of the immunogenicities and vaccine efficacies in mice of three recombinant forms of $DbpA_{N40}$ in various adjuvant formulations, and the in vitro potencies of their antisera

Expt[a]	Adjuvant[b]	Immunogen[c]	Two immunizations			Three immunizations		
			End-point titer		Infection prevalence[h]	End-point titer		Infection prevalence[h]
			DbpA IgG[d]	Growth inhibition[e]		DbpA IgG[d]	Growth inhibition[e]	
1	Freund's	Lpp:$DbpA_{N40}$	2,048,000	1,280	0/5[g]	ND[f]	ND	ND
		H_6DbpA_{N40}	1,024,000	<160	5/5	ND	ND	ND
		E. coli	128,00	<40	5/5	ND	ND	ND
2	Freund's	Lpp:$DbpA_{N40}H_6$	512,000	80	ND	2,048,000	ND	0/5[g]
		Lpp:$DbpA_{N40}$	256,000	<40	ND	512,000	ND	0/5[g]
		H_6DbpA_{N40}	512,000	ND	ND	512,000	ND	0/5[g]
		E. coli	4,000	ND	ND	2,000	ND	5/5
	TDM/MPL	Lpp:$DbpA_{N40}H_6$	256,000	40	ND	128,000	ND	0/5[g]
		Lpp:$DbpA_{N40}$	256,000	<40	ND	256,000	ND	2/5
		H_6DbpA_{N40}	8,000	ND	ND	256,000	ND	4/5
		E. coli	16,000	ND	ND	4,000	ND	5/5
3	Freund's	Lpp:$DbpA_{N40}H_6$	2,048,000	400	ND	1,024,000	2,560	0/5[g]
		Lpp:$DbpA_{N40}H_6$	512,000	100	ND	512,000	640	1/5[g]
		E. coli	4,000	<25	ND	32,000	<40	5/5
	Quil A	Lpp:$DbpA_{N40}H_6$	4,096,000	200	ND	4,096,000	>2,560	0/5[g]
		Lpp:$DbpA_{N40}$	2,048,000	<25	ND	1,024,000	640	0/5[g]
		E. coli	64,000	ND	ND	128,000	ND	5/5
	Alhydrogel	Lpp:$DbpA_{N40}H_6$	128,000	200	ND	128,000	2,560	0/5[g]
		Lpp:$DbpA_{N40}$	256,000	<25	ND	128,000	640	0/5[g]
		E. coli	4,000	ND	ND	2,000	ND	5/5
	Alhydrogel + Quil A	Lpp:$DbpA_{N40}H_6$	1,024,000	1,600	ND	256,000	>2,560	0/5[g]
		Lpp:$DbpA_{N40}$	4,096,000	<25	ND	512,000	640	1/5[g]
		E. coli	2,000	ND	ND	2,000	ND	5/5

[a] Immunizations were intraperitoneal in experiment 1 and subcutaneous in experiments 2 and 3.
[b] Freund's, complete Freund's adjuvant for first immunization and incomplete Freund's adjuvant thereafter, TDM/MPL, trehalose dimycolate plus monophosphoryl lipid A.
[c] Immunogens were 20 μg for DbpA or 5 μg for *E. coli* (negative control).
[d] Values are the means of duplicate ELISA titer determinations against Lpp:$DbpA_{N40}$ on antisera pooled from the five mice within each immunization group.
[e] Values are the means of duplicate inhibition titer determinations on antisera pooled from the five mice within each immunization group.
[f] ND not determined.
[g] $P < 0.05$ versus control (Fisher's exact test).
[h] Number infected/number tested.

some unidentified property of the amino-terminal sequence of H_6DbpA_{N40} did not influence the structure and function of DbpA.

Alterations to DbpA structure diminish protective immune responses. The above data suggested a correlation between the ability of the DbpA to bind to decorin and its ability to elicit a protective immune response in mice. If this is so, then perturbations of the protein structure that lead to a decrease in the ability to bind to decorin should also lead to a diminution in its potency as a protective immmunogen. In an attempt to generate such a functionally altered immunogen, we tested the ability of DbpA to bind to decorin following the unfolding of the protein by either heat denaturation or urea denaturation. As shown in Fig. 2B, the ability of the DbpA to bind to the decorin is retained in samples treated with 8 M urea but is lost on heat treatment at 65°C for 1 h.

DbpA from *B. burgdorferi* isolate 297 was shown by circular dichroism (CD) to have about 50 to 60% α-helical secondary structure (23), and we have found $DbpA_{N40}$ to be similar. We used CD to confirm the loss of Lpp:$DbpA_{N40}H_6$ secondary structure after denaturation by heat or in the presence of urea. Rapid removal of urea by dilution allowed the recovery of Lpp:$DbpA_{N40}H_6$ secondary structure (data not shown), as expected from the functional assay. Measured at ambient temperature, the secondary structures of Lpp:$DbpA_{N40}$ and $H_{10}DbpA_{N40}$ were substantially similar to that of Lpp:$DbpA_{N40}H_6$, suggesting that differences in tertiary structure may account for the functional and immunological differences among these proteins.

A comparison of the 30 DbpA sequences from various isolates of *B. burgdorferi* sensu lato determined by this laboratory revealed no obvious sequence motifs that are conserved among all DbpAs and might contribute to conserved structural elements (38). However, one or more conserved lysine residues distributed throughout DbpA have been shown by others to play a role in decorin binding activity (6). Of these 30 DbpA sequences, there are only 7 that contain cysteine residues other than the lipidation site amino-terminal cysteine. $DbpA_{N40}$ is of this type that contains two closely spaced carboxy-terminal cysteine residues. The presence of a putative disulfide bond in $DbpA_{N40}$ provided an opportunity to introduce an alteration to the tertiary structure of this protein that was less destructive than complete denaturation by heat or chemicals. To investigate the importance of these residues for the vaccine potency and structure of the N40 protein, we mutated these two cysteine codons in Lpp:$DbpA_{N40}H_6$ to serine. We compared the immune responses to the mutant Lpp:$DbpA_{N40}H_6$ (C176, 191S) with those to the unaltered and heat-denatured forms of

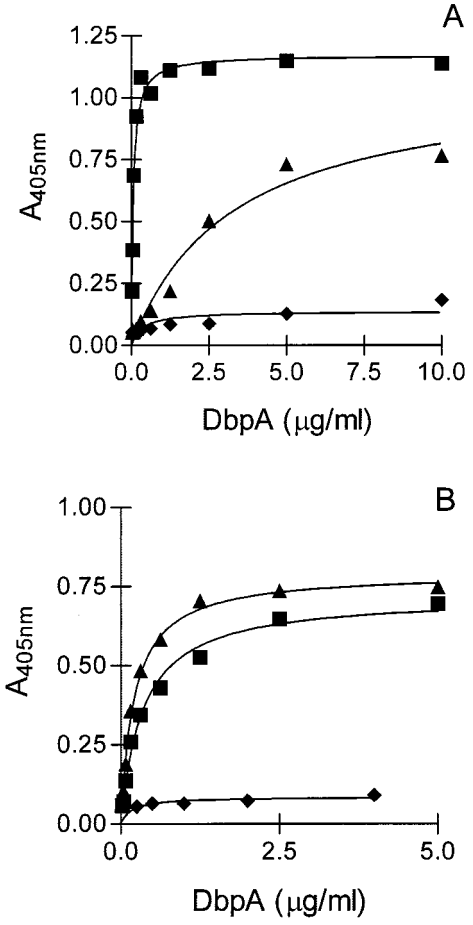

FIG. 2. Binding of different forms of $DbpA_{N40}$ to immobilized decorin. (A) Binding of Lpp:$DbpA_{N40}H_6$ (solid squares), Lpp:$DbpA_{N40}$ (solid triangles) and H_{10}-$DbpA_{N40}$ (solid diamonds) to decorin. (B) Binding of Lpp$DbpA_{N40}H_6$ (solid squares) treated with urea (solid triangles) or after heat treatment at 65°C for 1 h (solid diamonds). A_{405nm} absorbance at 405 nm.

this protein and to nonsecreted $H_{10}DbpA_{N40}$. Alhydrogel formulations of all four forms of $DbpA_{N40}$ elicited antisera with comparable specific IgG levels (Table 2). Both structurally altered forms of Lpp:$DbpA_{N40}H_6$ were inferior to the unaltered form in their ability to elicit borreliacidal antibodies and protection of mice from *B. burgdorferi* challenge. After two immunizations, $H_{10}DbpA_{N40}$ was intermediate in vaccine potency for borreliacidal antibodies and protection, and three immunizations were required for this antigenic form to elicit vaccine potency equivalent to that of Lpp:$DbpA_{N40}H_6$, similar to that seen with H_6DbpA_{N40} (Table 1). Unexpectedly, the mutant Lpp:$DbpA_{N40}H_6$ (C176, 191S) protein had decorin binding activity nearly equivalent to that of the unaltered form of Lpp:$DbpA_{N40}H_6$ (data not shown), suggesting that the (C176, 191S) mutant protein is able to fold into an active conformation, at least in the presence of its ligand. Additionally, we observed that recombinant DbpA from *B. burgdorferi* B31 remained effective as a vaccine after urea denaturation and renaturation (data not shown).

DbpA antibodies that are cross-reactive and borreliacidal for different *B. burgdorferi* sensu lato species are directed against conformational epitopes. We showed previously that antiserum against a single DbpA immunogen could kill diverse isolates of *B. burgdorferi, B. garinii*, and *B. afzelii* (27). These diverse isolates had substantial heterogeneity in their DbpA sequences, which prompted speculation that the epitopes binding the cross-reactive borreliacidal antibodies were composed of discontiguous amino acids (38). The availability of antisera against forms of $DbpA_{N40}$ that are conformationally and antigenically distinct allowed us to test this hypothesis directly.

For this analysis we screened representative isolates of *B. garinii* and *B. afzelii* that were divergent in primary structure from the N40 immunogen for their vulnerability to killing by antiserum against DbpA from *B. burgdorferi* N40. We determined that *B. garinii* isolate VSBP was killed by antiserum against Lpp2:$DbpA_{N40}H_6$, and $DbpA_{N40}$ has 52.6% identity to $DbpA_{VSBP}$ that we reported previously (38), so this isolate was selected as representative of this species.

B. afzelii strain PKo was also found to be vulnerable to Lpp2:$DbpA_{N40}H_6$ antiserum. To characterize the target for the borreliacidal antibodies, we cloned the *dbpA* gene from strain PKo using a PCR approach and primers that were previously successful for *dbpA* genes from other *B. afzelii* isolates (38). The putative *dbpA* gene from *B. afzelii* PKo was identical to the sequence reported for the recently described *osp17* gene from this same isolate (29). Osp17 was characterized as an immunodominant surface protein, but its function was not addressed in that study. The product of our candidate *dbpA* gene from *B. afzelii* PKo is 92.4% identical to the deduced DbpA sequence we reported previously for *B. afzelii* ACA1 and highly similar to several other *B. afzelii* DbpAs (38). To address whether *osp17* is equivalent to the *B. afzelii* PKo allele of *dbpA*, the recombinant product of our cloned *B. afzelii* gene encoding the putative $DbpA_{PKo}$ was compared to $DbpA_{N40}$ and $DbpA_{VSBP}$ in the decorin binding assay. $DbpA_{N40}$, $DbpA_{VSBP}$, and putative the $DbpA_{PKo}$ all bound to decorin, while the negative control protein, OspC, did not (Fig. 3). On the basis of our genetic and biochemical evidence, we conclude that $DbpA_{PKo}$ and $Osp17_{PKo}$ are the same protein, and we will use the DbpA nomenclature here. $DbpA_{N40}$ and $DbpA_{PKo}$ have 37.2% identity. Strain PKo was selected as representative of *B. afzelii*.

The antisera from the previous experiment evaluating altered forms of $DbpA_{N40}$ for vaccine effectiveness against the homologous strain (Table 2) were then tested for killing activity against the heterologous VSBP and PKo borreliae. Antiserum against Lpp2:$DbpA_{N40}H_6$ had borreliacidal activity against *B. garinii* VSBP and *B. afzelii* PKo, but antisera against the altered forms of this immunogen were inactive against both the homologous N40 strain and the heterologous VSBP and

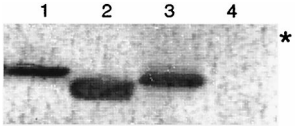

FIG. 3. Decorin binding activity blot. Protein samples on a polyvinylidene difluoride membrane were probed with biotinylated decorin and were visualized using a streptavidin-alkaline phosphatase conjugate using ECF reagent. Lanes: 1 Lpp:$DbpAN_{40}H_6$; 2, Lpp:$DbpA_{Pko}$; 3, Lpp:$DbpA_{VSBP}$-H_6; 4, $OspC_{N40}$.*, migration position of OspC.

TABLE 2. Comparison of the immunogenicities and vaccine efficacies in mice of physically or mutationally altered forms of $DbpA_{N40}$ adjuvanted with aluminum, and the in vitro potencies of their antisera

Immunogen[a]	Two immunizations			Three immunizations		
	End-point titer		Infection prevalence[e]	End-point titer		Infection prevalence[e]
	DbpA IgG[b]	Growth inhibition[c]		DbpA IgG[b]	Growth inhibition[c]	
Lpp:$DbpA_{N40}H_6$	32,000	200	1/5[d]	32,000	400	1/5[d]
Lpp:$DbpA_{N40}H_6$ 65°C	32,000	<50	4/5	32,000	<50	4/5
Lpp:$DbpA_{N40}H_6$(C176, 191S)	32,000	50	5/5	32,000	50	5/5
$H_{10}DbpA_{N40}$	32,000	100	2/5	32,000	400	1/5[d]
E. coli	1,000	<50	5/5	500	<50	5/5

[a] Immunogens, 10 μg for DbpA or 2.5 μg for *E. coli* (negative control), were formulated with Alhydrogel and injected subcutaneously. Lpp:$DbpA_{N40}H_6$ 65°C, Lpp:$DbpA_{N40}H_6$ heated for 60 min at 65°C. Lpp:$DbpA_{N40}H_6$(C176, 191S), Cys codons 176 and 191 of Lpp:$DbpA_{N40}H_6$ mutated to Ser.
[b] Values are the means of duplicate ELISA titer determinations against Lpp:$DbpA_{N40}$ on antisera pooled from the five mice within each immunization group.
[c] Values are the means of duplicate inhibition titer determinations on antisera pooled from the five mice within each immunization group.
[d] $P < 0.05$ versus control (Fisher's exact test).
[e] Number infected/number tested.

PKo strains (Table 3). Antiserum from mice immunized three times with $H_{10}DbpA_{N40}$ was similar to Lpp2:$DbpA_{N40}H_6$ antiserum for growth-inhibitory activity against the homologous N40 strain, but antiserum against the cytosolic form of $DbpA_{N40}$ was somewhat less effective against the heterologous strains.

Structural stability of different recombinant DbpA forms. The immunization experiments indicated a substantial difference in the epitope structures of Lpp:$DbpA_{N40}H_6$ and Lpp:$DbpA_{N40}H_6$(C176, 191S) that was not evident by CD or decorin binding assays. We next used the Arg-specific protease clostripain as a probe of the structural differences between Lpp:$DbpA_{N40}H_6$ and Lpp:$DbpA_{N40}H_6$(C176, 191S), reflected in a differential accessibility of their four Arg residues. Lpp:$DbpA_{N40}$ and $H_{10}DbpA_{N40}$ were also included in this comparison. At room temperature, incubation with 10% (wt/wt) clostripain resulted in a shift from the full-length Lpp:$DbpA_{N40}H_6$ molecule to a carboxy-terminally truncated form of the protein that was confirmed by Western blotting with a monoclonal antibody against the histidine tag. This single-cleavage product accounts for 50% of the remaining protein after 60 min of incubation (Fig. 4). In marked contrast, the (C176, 191S) mutant had lost 82% of the total protein after 15 min of incubation. The cysteine mutant DbpA protein was cleaved to a ~14-kDa limited digestion product which did not accumulate in the unaltered Lpp:$DbpA_{N40}H_6$ protein. At 37°C, the unaltered Lpp:$DbpA_{N40}H_6$ protein was nominally more sensitive to cleavage than at room temperature, whereas the cysteine mutant was almost completely degraded after 10 min of incubation. Lpp:$DbpA_{N40}$ and $H_{10}DbpA_{N40}$ showed only a limited sensitivity to clostripain digestion at either temperature, and a substantial amount of each full-length protein remained after the 60-min incubation. These data suggested that the cysteine mutant is less stable or structurally rigid than the unaltered Lpp:$DbpA_{N40}H_6$ protein and the other two forms of DbpA. The (C176, 191S) mutant protein was also the least effective vaccine antigen among these four.

To further assess the relative stability of the proteins, the effect of thermal denaturation on the secondary structure of the proteins was measured by CD. The CD spectrum of the cysteine mutant did not differ substantially from the unaltered Lpp:$DbpA_{N40}H_6$ protein at room temperature, demonstrating that these proteins have similar secondary structures (data not shown). Increasing the temperature caused a marked increase in the ellipticity at 220 nm. Comparison of the forms of the DbpA by thermal denaturation shows that the cysteine mutant form is less stable to increases in temperature than are Lpp:$DbpA_{N40}H_6$, Lpp:$DbpA_{N40}$, and H_{10}DbpAN40 proteins (Fig. 5). The cysteine mutant underwent what appeared to be a biphasic transition. There was a small change in ellipticity at 30°C, followed by a larger change in ellipticity at a T_m of 51°C. In contrast, the Lpp:$DbpA_{N40}H_6$ and the two other forms showed a structural transition at 65°C.

DISCUSSION:

Conformational B-cell epitopes contribute to the effectiveness of many vaccine antigens. Biochemical confirmation of native folding is not possible for antigens lacking a quantifiable function. With a few possible exceptions (14, 33), this is true of experimental and approved vaccines for *B. burgdorferi*. The biochemical activity of decorin binding proteins is measurable by numerous assays (23, 24) and appears to promote a physiologically relevant function, extracellular matrix adherence, for the borreliae. Point mutants of $DbpA_{297}$ have been made and tested for their ability to bind to decorin in various assay formats. These studies indicate lysine residues that may be important to decorin binding either directly or as a conse-

TABLE 3. Comparison of the effects of physical or mutational alterations to $DbpA_{N40}$ on its vaccine potency for in vitro growth inhibition activity against homologous and heterologous *B. burgdorferi* sensu lato isolates

Immunogen[a]	Antiserum growth inhibition titer against[b]:		
	B. burgdorferi N40	*B. garinii* VSBP	*B. afzelii* PKo
Lpp:$DbpA_{N40}H_6$	400	100	200
Lpp:$DbpA_{N40}H_6$ 65°C	<50	<50	<50
Lpp:$DbpA_{N40}H_6$(C176, 191S)	50	<50	<50
$H_{10}DbpA_{N40}$	400	50	100
E. coli	<50	<50	<50

[a] Immunogens, as described in Table 2, footnote *a*, were formulated with Alhydrogel and injected subcutaneously three times.
[b] Values are the means of duplicate inhibition titer determinations on antisera pooled from the five mice within each immunization group.

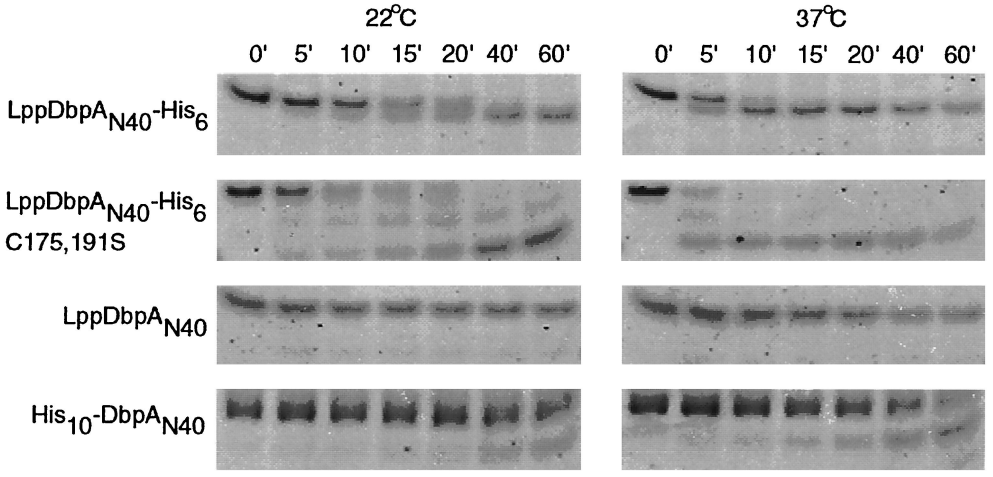

FIG. 4. Sensitivity of recombinant forms of DbpA$_{N40}$ to proteolysis. Proteins were incubated with 10% (wt/wt) clostripain at either 22 or 37°C. Aliquots were taken at the times indicated, and the reactions were quenched with 50 mM EDTA. Samples were run on a 10% Bis-Tris polyacrylamide gel in MES buffer, and proteins were visualized by Sypro Red staining.

quence of their affects on the conformation of the DbpA (6). We have now demonstrated further evidence of the importance of conformation in the activity of DbpA, and we have shown that eliciting a potent, protective immune response to DbpA required a properly folded form of the immunogen. Denaturation or mutations in the DbpA that lead to decreased thermal stability and increased protease sensitivity lead to an immune response that was not protective, suggesting that linear epitopes are less important for protection than are conformational epitopes. This is also consistent with the cross-reactivity of borreliacidal DbpA antibodies among divergent *B. burgdorferi* sensu lato isolates that lack candidates for conserved linear DbpA epitopes.

Recombinant DbpA expressed in the cytosol of *E. coli* with either of two different polyhistidine tags instead of the signal peptide was less effective as a vaccine antigen. These proteins were different structurally and functionally from secreted and acylated DbpA, suggesting that the subcellular compartment in which the antigen folds also influenced its antigenicity. Although we have not excluded the formal possibility that both of these polyhistidine tags altered an important protective antigenic epitope near the amino terminus of recombinant DbpA, we consider this unlikely since that end of the natural protein is proximal to the tripalmitoyl cysteine membrane anchor, which would limit its accessibility to antibodies on intact spirochetes. The production of DbpA as a lipoprotein has multiple advantages from a vaccine efficacy standpoint. First and foremost is that the DbpA as expressed in *Borrelia* is a secreted lipoprotein, and the expression of the DbpA as a recombinant lipoprotein should best maintain its normal context and post-translational processing. The folding of the DbpA in the periplasm is likely to be different from that in the cytoplasm, since the presence of chaperone proteins differs between these two compartments (31). Second, the lipid moiety serves as an integral adjuvant and as an activator of the immune response, which may improve the overall immunogenicity of the antigen. The effects of the cysteine-to-serine mutations on DbpA$_{N40}$ stability and antigenicity highlight the importance of the tertiary structure of the carboxy-terminal portion of the protein, presumably stabilized through a loop formed by a disulfide bond. In gram-negative bacteria and eukaryotes, disulfide bonds are catalyzed by extracytoplasmic oxidoreductases (37). Disulfide bonds in recombinant H$_6$DbpA$_{N40}$ and H$_{10}$DbpA$_{N40}$ may form spontaneously, albeit inefficiently, once they are released from the reducing environment of the *E. coli* cytosol, as has been shown to occur for the periplasmic enzyme alkaline phosphatase (11). Partial oxidation of cytosolic DbpA$_{N40}$ during purification, leading to the formation of the putative disulfide bond, would explain the intermediate vaccine efficacy of H$_6$DbpA$_{N40}$ and H$_{10}$DbpA$_{N40}$. Most DbpAs lack this carboxy-terminal cysteine pair and are presumably stabilized by other intrachain interactions.

Evidence for protective conformational epitopes has also been reported for other *B. burgdorferi* membrane proteins. Oms66 (or p66) is an outer membrane-spanning surface-exposed protein of *Borrelia* spp. that has recently been shown to be protective against infection by in vivo-adapted borreliae in a mouse model (14). This study showed that natural p66 anti-

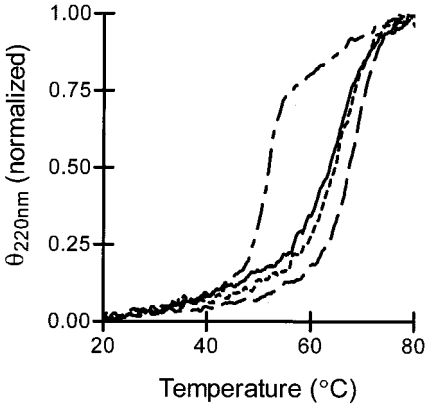

FIG. 5. CD thermal denaturation scans of four recombinant forms of DbpA$_{N40}$. Ellipticity was measured at 220 nm as a function of temperature for Lpp:DbpA$_{N40}$H$_6$ (——), Lpp:DbpA$_{N40}$ (- - - - -), H$_{10}$DbpA$_{N40}$ (– – – –), and Lpp:DbpA$_{N40}$H$_6$ (C176, 191S) (– - – - –).

gen isolated from *B. burgdorferi* had membrane channel-forming (porin) activity and was protective against the homologous isolate, but recombinant p66 produced in the *E. coli* cytosol as a GST fusion lacked porin activity and was not protective (14). OspC lipoprotein has also been shown to be an in vivo-expressed protective antigen; however, this protein is a protective immunogen against homologous challenge but not against heterologous challenge (5, 35, 36). OspC immunogen was shown to be sensitive to SDS or thermal denaturation (20, 21). The denatured forms of the OspC protein were not protective and did not elicit borreliacidal antibodies.

B. burgdorferi is unusual in its abundance of membrane lipoproteins, and several of these have shown substantial effectiveness as vaccines. However, even more putative or biochemically confirmed lipoproteins have shown partial or no protection. Most of these partially effective or apparently ineffective recombinant vaccine antigens have been expressed in *E. coli* as fusions to the cytosolic protein GST, including OspE and the related p21, OspF and the related pG, BmpA(P39), p30, p37, p55, BBK50/P37, and lp6.6 (4, 9, 10, 15, 16, 19, 30, 32, 43). It is possible that this convenient expression and purification strategy may have, in some cases, altered the folding of these otherwise-secreted proteins and compromised the vaccine efficacy of the recombinant products, as was the case with Oms66/p66 (14). Several *B. burgdorferi* lipoproteins expressed as GST fusions, including OspA, OspB, OspC, and p35/BBK32, have shown vaccine efficacy, however (18, 19, 45).

We have previously reported that Freund's adjuvant formulations of cytosolically expressed recombinant DbpA protected mice from *B. burgdorferi* challenge (27), and this was confirmed by others (17, 25). In vitro growth inhibition assays with antiserum against a cytosolic polyhistidine-tagged form DbpA failed to yield reproducible results in the study by Hagman et al. (25). The results of our present study suggest that the poor biological activity of this antiserum may have been due to their use of a suboptimal DbpA immunogen. We have now shown, by direct comparison, that secreted forms of DbpA are more effective than nonsecreted DbpA when formulated with Freund's or Ribi adjuvants. Secreted lipoprotein DbpA was also effective when formulated with Alhydrogel (this study), a clinically relevant adjuvant, or injected without adjuvant (28). Hagman et al. recently reported that a Freund's adjuvant formulation of cytosolic polyhistidine-tagged DbpA failed to elicit sterilizing immunity in mice against *B. burgdorferi* challenge by multiple tick bite (26). This study also reported evidence that DbpA targets for protective immunity are expressed by tick-borne *B. burgdorferi* only in the mammalian stage, unlike OspA and OspC, which are expressed by spirochetes within ticks and are targets for transmission-blocking immunity (13, 22). Previously we have shown that immunization with secreted lipoprotein DbpA elicits antibodies that can kill mammalian-stage spirochetes that are resistant to OspA antibody (7). A more conformationally correct form of the immunogen may achieve effective DbpA immunity against infection or disease caused by tick transmitted *B. burgdorferi*. Experiments are in progress to address this possibility. Recent observations by Ohnishi et al. (32a) reveal a previously unappreciated heterogeneity in the antigenic profile of the population of tick-borne *B. burgdorferi*. DbpA was not examined in that study. Even if DbpA is not sufficient as a monovalent subunit vaccine to prevent tick-borne infection, it may serve as a component of an effective multisubunit vaccine combined with other in vivo-expressed antigens or in combination with OspA (28).

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REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
2. **Barthold, S. W.** 1999. Specificity of infection-induced immunity among *Borrelia burgdorferi* senso lato species. Infect. Immun. **67:**36–42.
3. **Barthold, S. W., M. S. deSouza, J. L. Janotka, A. L. Smith, and D. H. Persing.** 1993. Chronic Lyme borreliosis in the laboratory mouse. Am. J. Pathol. **143:**959–971.
4. **Barthold, S. W., S. Feng, L. K. Bockenstedt, E. Fikrig, and K. Feen.** 1997. Protective and arthritis-resolving activity in sera of mice infected with *Borrelia burgdorferi*. Clin. Infect. Dis. **25:**S9–S17.
5. **Bockenstedt, L. K., E. Hodzic, S. Feng, K. W. Bourrel, A. M. de Silva, R. R. Montgomery, E. Fikrig, J. D. Radolf, and S. W. Barthold.** 1997. *Borrelia burgdorferi* strain-specific OspC-mediated immunity in mice. Infect. Immun. **65:**4661–4776.
6. **Brown, E. L., B. P. Guo, P. O'Neal, and M. Höök.** 1999. Adherence of *Borrelia burgdorferi*. Identification of critical lysine residues in DbpA required for decorin binding. J. Biol. Chem **274:**26272–26278.
7. **Cassatt, D., N. K. Patel, N. D. Ulbrandt, and M. S. Hanson.** 1998. DbpA, but not OspA, is expressed by *Borrelia burgdorferi* during spirochetemia and is a target for protective antibodies. Infect. Immun. **66:**5379–5387.
8. **Cinco, M., M. Ruscio, and F. Rapagna.** 2000. Evidence of dbps (decorin binding proteins) among European strains of *Borrelia burgdorferi* sensu lato and in the immune response of LB patient sera. FEMS Microbiol. Lett. **183:**111–114.
9. **Das, S., S. W. Barthold, S. Stocker Giles, R. R. Montgomery, S. R. Telford III, and E. Fikrig.** 1997. Temporal pattern of *Borrelia burgdorferi p21* expression in ticks and the mammalian host. J. Clin. Investig. **99:**987–995.
10. **Das, S., D. Shraga, C. Gannon, T. T. Lam, S. Feng, L. R. Brunet, S. R. Telford, III, S. W. Barthold, R. A. Flavell, and E. Fikrig.** 1996. Characterization of a 30-kDa *Borrelia burgdorferi* substrate-binding protein homologue. Res. Microbiol. **147:**739–751.
11. **Derman, A. I., and J. Beckwith.** 1995. *Escherichia coli* alkaline phosphatase localized to the cytoplasm slowly aquires enzymatic activity in cells whose growth has been suspended: a caution for gene fusion studies. J. Bacteriol. **177:**3764–3770.
12. **de Silva, A., N. S. Zeidner, Y. Zhang, M. C. Dolan, J. Piesman, and E. Fikrig.** 1999. Influence of outer surface protein A antibody on *Borrelia burgdorferi* within feeding ticks. Infect. Immun. **67:**30–35.
13. **de Silva, A. M., S. R. Telford III, L. R. Brunet, S. W. Barthold, and E. Fikrig.** 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. **183:**271–275.
14. **Exner M. M., D. R. Ianco, J. N. Miller, and M. A. Lovett.** 2000. Protection elicited by native outer membrane protein Oms66 (p66) against host-adapted *Borrelia burgdorferi*: conformational nature of bactericidal epitopes. Infect. Immun. **68:**2647–2654.
15. **Feng, S., S. W. Barthold, S. R. Telford III, and E. Fikrig.** 1996. p55, an immunogenic but nonprotective 55-kilodalton *Borrelia burgdorferi* protein in murine Lyme disease. Infect. Immun. **64:**363–365.
16. **Feng, S., E. Hodzic, and S. W. Barthold.** 2000. Lyme arthritis resolution with antiserum to a 37-kilodalton *Borrelia burgdorferi* protein. Infect. Immun. **68:**4169–4173.
17. **Feng, S., E. Hodzic, B. Stevenson, and S. W. Barthold.** 1998. Humoral immunity to *Borrelia burgdorferi* N40 decorin binding proteins during infection of laboratory mice. Infect. Immun. **66:**2827–2835.
18. **Fikrig, E., S. W. Barthold, N. Marcantonio, K. Deponte, F. S. Kantor, and R. A. Flavell.** 1992. Roles of OspA, OspB, and flagellin in protective immunity to Lyme borreliosis in laboratory mice. Infect. Immun. **60:**657–661.
19. **Fikrig, E., W. Feng, S. W. Barthold, S. R. Telford, and R. A. Flavell.** 2000. Arthropod- and host-specific *Borrelia burgdorferi* bbk32 expression and the inhibition of spirochete transmission. J. Immunol. **164:**5344–5351.
20. **Gilmore, R. D. J., K. J. Kappel, M. C. Dolan, T. R. Burkot, and B. J. B. Johnson.** 1996. Outer surface protein C (OspC), but not p39, is a protective immunogen against a tick-transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC. Infect. Immun. **64:**2234–2239.

21. **Gilmore, R. D. J., and M. L. Mbow.** 1999. Conformational nature of the *Borrelia burgdorferi* B31 outer surface protein C protective epitope. Infect. Immun. **67:**5463–5469.
22. **Gilmore, R. D. J., and J. Piesman.** 2000. Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding of ticks on OspC-immunized mice. Infect. Immun. **68:**411–414.
23. **Guo, B. P., E. L. Brown, D. W. Dorward, L. C. Rosenberg, and M. Höök.** 1998. Decorin-binding adhesins from *Borrelia burgdorferi*. Mol. Microbiol. **30:**711–723.
24. **Guo, B. P., S. J. Norris, L. C. Rosenberg, and M. Höök.** 1995. Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. Infect. Immun. **63:**3467–3472.
25. **Hagman, K. E., P. Lahdenne, T. G. Popova, S. F. Porcella, D. R. Akins, J. D. Radolf, and M. V. Norgard.** 1998. Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two-gene operon and is protective in the murine model of Lyme borreliosis. Infect. Immun. **66:**2674–2683.
26. **Hagman, K. E., X. Yang, S. K. Wikel, G. B. Schoeler, M. J. Caimano, J. D. Radolf, and M. V. Norgard.** 2000. Decorin-binding protein A (DbpA) of *Borrelia burgdorferi* is not protective when immunized mice are challenged via tick Infection and correlates with the lack of DbpA expression by *B. burgdorferi* in ticks. Infect. Immun. **68:**4759–4764.
27. **Hanson, M. S., D. Cassatt, B. P. Guo, N. K. Patel, M. McCarthy, D. W. Dorward, and M. Höök.** 1998. Active and passive immunity against *Borrelia burgdorferi* decorin binding protein A (DbpA) protects against infection. Infect. Immun. **66:**2143–2153.
28. **Hanson, M. S., N. K. Patel, D. R. Cassatt, and N. D. Ulbrandt.** 2000. Evidence for vaccine synergy between *Borrelia burgdorferi* decorin binding protein A and outer surface protein A in the mouse model of Lyme borreliosis. Infect. Immun. **68:**6457–6460.
29. **Jauris-Heipke, S., B. Roble, G. Wanner, C. Habermann, D. Rossler, V. Fingerle, G. Lehnert, R. Lobentanzer, I. Pradel, B. Hillenbrand, U. Schulte-Spechtel, and B. Wilske.** 1999. Osp17, a novel immunodominant outer surface protein of *Borrelia afzelii*: recombinant expression in *Escherchia coli* and its use as a diagnostic antigen for serodiagnosis of Lyme borreliosis. Med. Microbiol. Immunol. **187:**213–219.
30. **Lahdenne, P., S. F. Porcella, K. E. Hagman, D. R. Akins, T. G. Popova, D. L. Cox, L. I. Katona, J. D. Radolf, and M. V. Norgard.** 1997. Molecular characterization of a 6.6-kilodalton *Borrelia burgdorferi* outer membrane-associated lipoprotein (lp6.6) which appears to be downregulated during mammalian infection. Infect. Immun. **65:**412–421.
31. **Missiakas, D., and S. Raina.** 1997. Protein folding in the bacterial periplasm. J. Bacteriol. **179:**2465–2471.
32. **Nguyen, T. P., T. T. Lam, S. W. Barthold, S. R. Telford, III, R. A. Flavell, and E. Fikrig.** 1994. Partial destruction of *Borrelia burgdorferi* within ticks that engorged on OspE- or OspF-immunized mice. Infect. Immun. **62:**2079–2084.
32a. **Ohnishi, J., J. Piesman, and A. M. de Silva.** 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. Proc. Natl. Acad. Sci. USA **98:**670–675.
33. **Pal, U., A. de Silva, R. R. Montgomery, D. Fish, J. Anguita, J. F. Anderson, Y. Lobet, and E. Fikrig.** 2000. Attachment of *Borrelia burgdorferi* within *Ixodes scapularis* mediated by outer surface protein A. J. Clin. Investig. **106:**561–569.
34. **Preac-Mursic, V., B. Wilske, E. Patsouris, S. Jauris, G. Will, S. Rainhardt, G. Lehnert, U. Klockmann, and P. Mehraein.** 1992. Active immunization with pC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection **20:**342.
35. **Probert, W. S., M. Crawford, R. B. Cadiz, and R. B. LeFebvre.** 1997. Immunization with outer surface protein (Osp) A, but not OspC, provides cross-protection of mice challenged with North American isolates of *Borrelia burgdorferi*. J. Infect. Dis. **175:**400–405.
36. **Probert, W. S., and R. B. LeFebvre.** 1994. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect. Immun. **62:**1920–1926.
37. **Raina, S., and D. Missiakas.** 1997. Making and breaking disulfide bonds. Annu. Rev. Microbiol. **51:**179–202.
38. **Roberts, W. C., B. A. Mullikin, R. Lathigra, and M. S. Hanson.** 1998. Molecular analysis of sequence heterogeneity among genes encoding decorin binding proteins A and B of *Borrelia burgdorferi* sensu lato. Infect. Immun. **66:**5275–5285.
39. **Santra, M., D. M. Mann, E. W. Mercer, T. Skorski, B. Calabretta, and R. V. Iozzo.** 1997. Ectopic expression of decorin protein core causes a generalized growth suppression in neoplastic cells of various histogenetic origin and requires endogenous p21, an inhibitor of cyclin-dependent kinases. J. Clin. Investig. **100:**149–157.
40. **Sigal, L. H., J. M. Zahradnik, P. Lavin, S. J. Patella, G. Bryant, R. Haselby, E. Hilton, M. Kunkei, D. Adler-Klein, T. Doherty, J. Evans, S. E. Malawista, and The Recombinant Outer-Surface Protein A Lyme Disease Vaccine Study Consortium.** 1998. A vaccine consisting of recombinant *Borrelia burgdorferi* outer-surface protein A to prevent Lyme disease. N. Engl. J. Med. **339:**216–222.
41. **Steere, A. C.** 1994. Lyme disease: a growing threat to urban populations. Proc. Natl. Acad. Sci. USA **91:**2378–2383.
42. **Steere, A. C., V. K. Sikand, F. Meurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. H. Schmid, S. Laukamp, C. Buscarino, D. Krause, and The Lyme Disease Vaccine Study Group.** 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. N. Engl. J. Med. **339:**209–215.
43. **Wallich, R., C. Brenner, M. D. Kramer, and M. M. Simon.** 1995. Molecular cloning and immunological characterization of a novel linear-plasmid-encoded gene, pG, of *Borrelia burgdorferi* expressed only in vivo. Infect. Immun. **63:**3327–3335.
44. **Xu, Y., and R. C. Johnson.** 1995. Analysis and comparison of plasmid profiles of *Borrelia burgdorferi* sensu lato strains. J. Clin. Microbiol. **33:**2679–2685.
45. **Zhong, W., L. Gern, T. Stehle, C. Museteanu, M. D. Kramer, R. Wallich, and M. M. Simon.** 1999. Resolution of experimental and tick-borne *Borrelia burgdorferi* infection in mice by passive, but not active immunization using recombinant OspC. Eur. J. Immunol. **29:**946–957.

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