Disulfide-Mediated Oligomer Formation in *Borrelia burgdorferi* Outer Surface Protein C, a Critical Virulence Factor and Potential Lyme Disease Vaccine Candidate

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*Borrelia burgdorferi* OspC is an outer membrane lipoprotein required for the establishment of infection in mammals. Due to its universal distribution among *B. burgdorferi* sensu lato strains and high antigenicity, it is being explored for the development of a next-generation Lyme disease vaccine. An understanding of the surface presentation of OspC will facilitate efforts to maximize its potential as a vaccine candidate. OspC forms homodimers at the cell surface, and it has been hypothesized that it may also form oligomeric arrays. Here, we employ site-directed mutagenesis to test the hypothesis that interdimeric disulfide bonds at cysteine 130 (C130) mediate oligomerization. *B. burgdorferi* B31 ospC was replaced with a C130A substitution mutant to yield strain B31::ospC(C130A). Recombinant protein was also generated. Disulfide-bond-dependent oligomer formation was demonstrated and determined to be dependent on C130. Oligomerization was not required for in vivo function, as B31::ospC(C130A) retained infectivity and disseminated normally. The total IgG response and the induced isotype pattern were similar between mice infected with untransformed B31 and those infected with the B31::ospC(C130A) strain. These data indicate that the immune response to OspC is not significantly altered by formation of OspC oligomers, a finding that has significant implications in Lyme disease vaccine design.

Lyme borreliosis is an emerging infectious disease in North America and Europe caused by the spirochetes *Borrelia burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii* (2, 3, 9, 21, 25, 31). *B. burgdorferi* is maintained in an enzootic cycle involving ixodes ticks and reservoir mammals and birds (4, 5, 18). As the spirochetes transit between ticks and reservoir hosts, differences in gene expression aids in adaptation to the radically different environments. OspC, a 21-kDa plasmid-encoded lipoprotein, is upregulated in ticks concurrent with the blood meal and is expressed at a high level during the first weeks of infection in mammals (6, 17, 19, 30, 42, 43). OspC is required to establish infection but not for persistence (20, 46–49). The function of OspC has not yet been clearly defined (37). It has been hypothesized that ligand binding domain 1 (LBD1) of OspC binds a small ligand and that this interaction is required for the establishment of infection in mammals (13). OspC also binds other ligands, including Salp15 (a tick-derived protein with immunomodulatory activity) and plasminogen, by unknown mechanisms (1, 10, 22, 23, 29, 38).

OspC is predominantly helical and forms a homodimer tethered to the outer membrane by an N-terminal tripalmitoyl-S-glyceryl-cysteine moiety (8, 11, 16, 17, 24, 27, 52). Residues lining the dimeric interface are conserved, while the remainder of OspC is variable in sequence. More than 30 phylogenetically distinct types of OspC have been defined (4, 11, 51). It has been hypothesized that OspC and its relapsing fever *Borrelia* ortholog (Vsp) form arrays in the outer membrane (30, 52).

OspC is a candidate for a next-generation Lyme disease vaccine (11, 12, 15, 28). The surface presentation of OspC may have implications for vaccine design. Array formation can influence the exposure of epitopes and induce T-cell-independent humoral immune responses (30). Here, we test the hypothesis that cysteine 130 (C130) forms an interdimeric disulfide bond that mediates the formation of higher-order oligomers or arrays.

MATERIALS AND METHODS

Site-directed mutagenesis and production of r-OspC and anti-OspC antiserum. Recombinant OspC (r-OspC) was produced by amplification of ospC (lacking the signal sequence and the lipidated cysteine) from wild-type (*wt*) B. burgdorferi strain B31 (type A OspC). A cysteine-to-alanine mutation at amino acid 130 (C130A) was accomplished by overlap extension/amplification PCR using mutagenic primers (5′-CGGCTAAAAAGCGTTCTGAACAC-3′ and its reverse complement) (12). Wild-type and C130A-mutated ospC genes were annealed to the pET46 Ek/LIC vector (Novagen), transformed into *Escherichia coli* (14), and confirmed by DNA sequencing (MWG Biotech). Recombinant proteins were expressed from *E. coli* BL21(DE3) cells and purified by nickel affinity chromatography (14). Anti-OspC antiserum was generated by immunization of C3H/HeJ mice with r-OspC (wt) adsorbed to alum (Imject, Pierce) by established protocols (13). Far-UV circular dichroism (CD) spectra of r-OspC (wt) and r-OspC(C130A) proteins were measured at 20°C in a Jasco J-715 spectropolarimeter (Jasco, Easton, MD) as previously described (13). Three independent scans were made of each protein.

Allelic exchange replacement of wild-type *ospC*. A pCAE1 allelic exchange vector was created which contained the *ospC*(C130A) gene (13). *ospC*(C130A) was amplified with 5′ and 3′ BspEI restriction sites, digested, and ligated into the BspEl-digested, dephosphorylated (CIP) pCAE1 vector. The plasmid was propagated in *Novabiolab E. coli* and purified (Qiagen), and the insert was sequenced (MWG Biotech). *B. burgdorferi* B31 clone 5A4 (35) was transformed as described previously (13), with selection by streptomycin. Clonal cultures of the resultant B31::ospC(C130A) strain were generated by subsurface plating, with plasmid content assessed by PCR using plasmid-specific primer sets (39). The
correct allelic exchange sequence was confirmed as previously described (13). Untransformed wild-type cells (B31), an ospC deletion mutant (B31::ospC(C130A)), and an allelic exchanged strain carrying the wild-type ospC gene [B31::ospC(wt)] served as controls (13). Growth rates were determined by daily triplicate cell counts of cultures grown at 37°C in Barbour-Stoenner-Kelly (BSK) medium (no antibiotics).

**Proteasine K digestion and immunofluorescence assays.** The presentation of OspC at the Borrelia cell surface was assessed by immunofluorescent antibody (IFA) assay and treatment of intact cells with proteasine K. Cells were transferred from 33°C to 37°C (and maintained for 2 days) to upregulate OspC production. Surface exposure of proteins was assessed by proteasine K digestion and Western blotting as previously described, with periplasmic FlaB protein serving as a control (13). To assess the distribution of OspC at the cell surface, immobilized cells were probed with anti-OspC antisera (1:2,000) and Alexa 488-conjugated goat anti-mouse IgG (1:200) (13). Slides were mounted with Prolong Gold (Invitrogen), and the cells were visualized using an Olympus BX51 fluorescence microscope.

**Analysis of disulfide-bond-mediated oligomerization.** Interdimeric disulfide bond formation was assessed in r-OspC(wt) and r-OspC(C130A), and in OspC expressed by the B31, B31::ospC(wt), and B31::ospC(C130A) strains, with B31::ospC as a negative control. All Borrelia strains were transferred to 37°C for 2 days prior to harvesting to upregulate OspC expression (6, 45). The cells were washed twice with phosphate-buffered saline (PBS), suspended to an optical density at 600 nm (OD600) of 0.002 µl−1 in reducing (β-mercaptoethanol) or nonreducing SDS-PAGE sample buffer, and incubated at 100°C for 10 min. r-OspC (30 ng) and 2 µl of each cell lysate were separated by SDS-PAGE, blotted, and probed with anti-OspC antisera (1:2,000) and goat anti-mouse IgG-HRP (1:40,000). Molecular masses of the OspC bands were estimated by interpolation from a standard curve generated with NativeMark (Invitrogen) and bovine and chicken egg albumin.

**Assessment of plasmidinogen binding by wild-type and site-directed mutant proteins.** Plasmidinogen binding by recombinant OspC was assessed by enzyme-linked immunosorbent assay (ELISA) (29). ELISA plates (Costar 3590) were coated with plasmidinogen (P7999; 500 ng well−1 [Sigma] in carbonate buffer [pH 9.6]). The wells were blocked with 1% bovine serum albumin (BSA) in PBS-Tween (PBS-T), r-OspC proteins (1 µg well−1 in blocking buffer) were incubated in triplicate wells for 2 h at room temperature. Bound OspC was detected by mouse anti-His tag monoclonal antibody (MAB, 1:1,000; Pierce) and then by goat anti-mouse IgG-HRP. Binding was quantified with ABTS (2,2' azinobis(3-ethylbenzthiazolinesulfonic acid) chromogen).

**Mouse infection.** To assess infectivity and dissemination, mice (two trials of five C3H/HeJ mice per group) were needle inoculated with 106 cells of B31, B31::ospC(wt), B31::ospC(C130A), or B31::ospC(C130A). After 4 weeks, the mice were bled by tail nick and euthanized, and tissues were collected from the ear and bladder (five mice) or the ear, bladder, brain, heart, tibiotarsal joint, and kidney (five mice). Tissues were placed in BSK medium supplemented with rifampin, fusidic acid, and amphotericin B. The IgG response was determined in all mice by ELISA, with immobilized whole B. burgdorferi cells or r-OspC serving as the capture antigen, using standard methods as previously described (13). Potential differences in induced antibody isotypes were assessed by ELISA. ELISA plates were coated with r-OspC(wt) at 200 ng well−1, blocked with 1% BSA in PBS-T, and then probed with a 1:100 dilution of sera from mice infected with B31 or B31::ospC(C130A) strains. Secondary detection was by mouse isotype-specific goat antiserum (Sigma) and rabbit anti-goat IgG-HRP.

**RESULTS**

Production of C130A mutant r-OspC and analysis of protein secondary structure. Full-length native OspC harbors two Cys residues: an N-terminal tripalmitoyl glyceryl-modified Cys and C130. r-OspC was generated that lacked the leader sequence and the N-terminal Cys residue. In the native protein, the modification of the N-terminal Cys prevents it from playing a role in disulfide-bond-mediated oligomerization. To determine if C130 is involved in OspC oligomerization, recombinant protein harboring a C130A substitution, r-OspC(C130A), was generated. Circular dichroism revealed alpha-helical contents of 56% ± 4% and 57.5% ± 4% for r-OspC(C130A) and r-OspC(wt), respectively.

**Allelic exchange of the ospC gene and analysis of the resulting strains.** To determine if OspC dimers form disulfide bonds that mediate higher-order oligomerization in vivo, B. burgdorferi B31 ospC was replaced with ospC(C130A) by allelic exchange. Control strains included B31::ospC(wt) (a wild-type ospC gene replaced with a wild-type ospC gene flanked by a resistance cassette) and B31ΔospC (ospC deletion mutant) (13). The B31::ospC(C130A) and B31ΔospC strains contain all parental plasmids, while the B31::ospC(wt) strain lacks lp21 (13). Loss of lp21 is inconsequential as it is not required for infectivity (35). The production of OspC(C130A) and the expression of antibiotic resistance by B. burgdorferi B31 did not affect growth rate (data not shown). While immunoblot analyses of the in vitro–cultivated strains suggest minor differences in OspC expression, the resulting anti-OspC titers in mice were similar (discussed below). It is important to note that only a subset of cells produce OspC during cultivation (44). Hence, the expression differences among cultivated strains are most likely a reflection of this phenomenon.

**Analysis of OspC production and surface presentation.** OspC production in all strains (except B31ΔospC) was demonstrated by immunoblot analysis (Fig. 1A). To determine if each strain presents OspC at the cell surface in a manner consistent with the parental B31 strain, proteasine K digestion and IFA analyses were performed. Cells were exposed (or not exposed) to proteasine K, and immunoblots of the cell lysates were screened with anti-OspC or anti-FlaB antisera. Exposure to proteasine K resulted in the loss of detection of OspC, but not the periplasmic FlaB (Fig. 1A). IFA demonstrated no visible differences in surface labeling patterns (Fig. 1B). It can be concluded that the C130A substitution does not influence the surface presentation of OspC.

**Assessment of oligomerization of OspC in vitro and in vivo.** To assess the formation of disulfide bonds, r-OspC proteins and cell lysates of each strain were analyzed by SDS-PAGE under reducing (β-mercaptoethanol) and nonreducing conditions (Fig. 2A). The monomeric native OspC and His-tagged r-OspC proteins have masses of 20.3 kDa and 22 kDa, respectively. Under nonreducing conditions, r-OspC(wt) existed in both monomeric and dimeric forms, while r-OspC(C130A) existed only in monomeric form (Fig. 2A). Under reducing conditions, only monomers were detected (see the conceptual model presented in Fig. 2C to F). Dimeric OspC was also detected in B31 and B31::ospC(wt) cell lysates when separated under nonreducing conditions but not in the B31::ospC(C130A) cell lysate.

To determine if r-OspC dimers can oligomerize, r-OspC(wt) and r-OspC(C130A) were separated under nondenaturing (both reducing and nonreducing) conditions using blue native PAGE. Under nonreducing conditions, five immunoreactive
In mice was assessed using needle inoculation in two trials (five mice per group). Four weeks postinoculation, blood was collected and tissue biopsy samples were harvested and placed in medium. Positive cultures were found in all mice, except those infected with the negative control B31::ospC strain. Cultivation of each infectious strain from mouse tissues did not reveal differences in dissemination patterns among strains (Table 1).

To assess the antibody response elicited by each strain, anti-OspC and anti-B. burgdorferi IgG titers were determined by ELISA using r-OspC or B. burgdorferi whole cells as the immobilized antigen. Whole-cell and OspC-specific IgG titers were similar for mice infected with B31, B31::ospC(wt), and B31::ospC(C130A) cells (Fig. 3). In addition, no significant differences were detected in the OspC-specific isotype profile in mice infected with B31 or B31::ospC(C130A) (Fig. 4).

**DISCUSSION**

OspC is an important virulence factor for species of the B. burgdorferi sensu lato complex and a potential candidate for a next-generation Lyme disease vaccine (12, 14, 15, 20, 34, 46–49). Efforts to define the function of OspC and develop a vaccine have been significantly enhanced by recent analyses of OspC structure-function relationships and its antigenic structure (13, 16, 26, 27, 51). A postulate that has not yet been tested is that OspC may form functionally and immunologically significant arrays in the *Borrelia* outer membrane (30). While freeze-fracture microscopic analyses indicate the existence of membrane protein arrays in B. burgdorferi, the composition of these arrays have not yet been determined (36). Here we test the hypothesis that hydrophobic based OspC dimers form biologically relevant higher-order oligomers that result from interdimeric disulfide bonding mediated by residue C130. To assess this, a recombinant site-directed OspC mutant protein, r-OspC(C130A), and a B. burgdorferi strain that produces this site-directed mutant, B31::ospC(C130A), were generated and OspC oligomerization tested. Recombinant wild-type OspC formed oligomeric chains of two to five dimers. Formation of these oligomers was eliminated by reducing conditions and proved to be strictly dependent on C130. While technical limitations prevented the direct assessment of OspC oligomeric length at the *Borrelia* cell surface, the occurrence of disulfide-bonded OspC at the cell surface and the data obtained with recombinant proteins support the notion that disulfide-dependent oligomers form *in vivo*.

Direct functional assays for OspC have not yet been defined. However, due to the essential nature of OspC, mutations that perturb critical determinants of the protein can be identified by assessing infectivity in mice. To determine if OspC oligomerization is required for OspC to carry out its *in vivo* function, wild-type and B31::ospC(C130A) strains were inoculated into mice and infectivity and dissemination assessed. While substitution of C130 with Ala prevents higher-order oligomerization, it did not abolish infectivity as inferred by seroconversion and the ability to cultivate spirochetes from ear punch biopsies of inoculated mice. Dissemination was assessed by cultivation of biopsy samples harvested at sites distal from the original inoculation site. An apparent difference in dissemination potential was not observed. These analyses definitively demonstrate that disulfide-bond-mediated oligomerization is not required for survival and dissemination within the mammalian host.
Recent efforts from our laboratory to generate a broadly protective, recombinant chimeric OspC-derived vaccine have focused on the generation of a chimera consisting primarily of the loop 5-helix 3 junction from diverse strains. This region of OspC has been shown to harbor linear epitopes that elicit bactericidal antibody (7, 11). The highly conserved C130 residue resides at the N-terminal end of this antigenic region. C130-mediated oligomerization and array formation at the cell surface could induce a T-independent humoral response, potentially impacting the memory response of vaccinees during infection. To assess this, serum was collected from the mice.

![FIG. 2. Formation of disulfide-dependent oligomers by r-OspC and OspC expressed at the Borrelia cell surface.](image)

**TABLE 1. Summary of culture results from tissues of mice inoculated with B31 and ospC transgenic strains**

| Strain         | Ear | Bladder | Brain | Heart | Joint | Kidney |
|----------------|-----|---------|-------|-------|-------|--------|
| B31 (untransformed) | 10/10 | 10/10 | 1/5   | 3/5   | 1/5   | 1/5    |
| B31::ospC(wt)    | 10/10 | 9/10   | 0/5   | 5/5   | 3/5   | 0/5    |
| B31::ospC(C130A) | 5/10  | 10/10  | 0/5   | 4/5   | 2/5   | 0/5    |
| B31ΔospC        | 0/10  | 0/10   | 0/5   | 0/5   | 0/5   | 0/5    |

![FIG. 3. Analysis of the antibody response to B. burgdorferi B31 and ospC transgenic strains in mice.](image)

The hydrophobically bound OspC dimer (C) can be maintained under native conditions or separated into component monomers (D) under denaturing conditions. In contrast, an oligomer composed of two or more dimers covalently bound at C130 (E) will dissociate under denaturing conditions into monomers and covalently bound dimers (F), which will dissociate to monomers only under reducing conditions. All models are derived from structure 1GGQ (27).
FIG. 4. Anti-OspC antibody isotype profile in mice infected with B. burgdorferi B31 or B31::ospC(C130A). ELISAs were conducted using serum harvested from mice 4 weeks after needle inoculation. Antibodies were captured by immobilized r-OspC(Wt) and detected by isotype-specific antisera. Columns indicate the mean absorbance, with standard deviations between mice indicated by the error bars.

infected with each strain, and the IgG titer and antibody isotype patterns were determined. Titer and antibody isotype patterns were the same for mice infected with the wild-type strain and C130A substitution mutant strain. These data indicate that disulfide bond-mediated oligomerization does not significantly influence immune responses to OspC. The data also demonstrate that presentation of a potentially repetitive epitope, such as that which would be expected in an OspC higher-order oligomeric array, does not induce a type 2 T-cell-independent immune response (33).

In summary, it can be concluded that while OspC forms oligomers, disulfide-bond-mediated oligomerization is not required for OspC to function in the establishment of infection, dissemination, or the generation of a robust and potential productive antibody response.

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