Characterization of an anti-*Borrelia burgdorferi* OspA conformational epitope by limited proteolysis of monoclonal antibody-bound antigen and mass spectrometric peptide mapping

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Abstract

Lyme borreliosis is a multisystem disorder caused by the spirochete *Borrelia burgdorferi* that is transmitted to humans by the tick *Ixodes dammini*. The immune response against the 31 kDa OspA, which is one of the most abundant *B. burgdorferi* proteins, appears to be critical in preventing infection and tissue inflammation. Detailed knowledge of the immunological and molecular characteristics of the OspA protein is important for the development of reliable diagnostic assays. In this study, we characterized a new conformational epitope present within the middle part of *B. burgdorferi* OspA. Our approach used enzymatic proteolyses of the immune complex followed by mass spectrometric identification of the peptides bound to the antibody. It appears to be one of the first reports on the characterization of a discontinuous epitope using mass spectrometry.

Keywords: *Borrelia burgdorferi*; discontinuous epitope; Lyme disease; mass spectrometry; monoclonal antibody

Lyme borreliosis is a multisystem disorder caused by the spirochete *Borrelia burgdorferi* that is transmitted to humans by the tick *Ixodes dammini* (Burgdorfer et al., 1982; Steere, 1989). Among the many antigens recognized by sera from infected humans, three spirochetal membrane proteins are recognized in a defined temporal sequence. Immunoglobulins to the 41 kDa flagellin protein appear early in the infection, whereas antibody response to the outer surface proteins A and B (OspA and OspB) develops later (Craft et al., 1986; Dattwyler & Luft, 1989).

The immune response against the 31 kDa OspA, which is one of the most abundant *B. burgdorferi* proteins, appears to be critical in preventing infection and tissue inflammation (Schaible et al., 1990). OspA anchors in the outer membrane via a lipidated N-terminal cysteine (Brandt et al., 1990). However, recombinant unlipidated

OspA was recognized by sera from patients with Lyme disease (Dunn et al., 1990), and further different studies suggested that recombinant OspA could be used to vaccinate humans against infection with *B. burgdorferi* spirochete (Fikrig et al., 1990; Simon et al., 1991). Recently, effective and safe OspA vaccines, with or without adjuvant, were released (Sigal, 1999; Wahlberg, 1999). Hence, detailed knowledge of the immunological and molecular characteristics of OspA protein is important for the development of reliable diagnostic assays.

Monoclonal antibodies (mAbs) to OspA that recognize specific amino- and carboxy-terminal antigenic determinants have been reported in previous studies (Schubach et al., 1991; Sears et al., 1991; Wilske et al., 1991). Moreover, epitope mapping studies using chemically cleaved OspA and mAbs have indicated that the region flanking the tryptophan at residue 200 is hypervariable. Specific amino acid changes near the tryptophan can abolish the reactivity of OspA to these monoclonal antibodies (France et al., 1993; McGrath et al., 1995). In humans, anti-OspA antibodies produced during natural infection also recognize the carboxy-terminal portion of the OspA protein but do not recognize the epitopes that bind mouse mAbs (Schubach et al., 1991; Shanafelt et al., 1992). However, the C-terminal domain is the target of most agglutinating and neutralizing antibodies directed against OspA

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Abbreviations: B/U ratio, bound/unbound ratio; CNBr, cyanogen bromide; DEA, diethylamine; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; mAb, monoclonal antibody; MS, mass spectrometry; OspA, outer surface protein A; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIC, total ion current.

(Sears et al., 1991; Wilske et al., 1992). Recently, biophysical studies including crystal structure (Li et al., 1997) and NMR (Huang et al., 1998) of OspA-mAb Fab complexes allowed localization of two mAb specific epitopes on the three-dimensional (3D) structure of OspA.

In the last 10 years, mass spectrometry has become a powerful tool in biological research. As a central technique integrated within a global method, mass spectrometry enables a rather fast characterization of the covalent structures of proteins (sequence, post-translational modifications, etc.) while consuming small amounts of samples. Furthermore, specific strategies are being developed to probe noncovalent structural features, in particular protein-protein interactions. Some of these methods have already been used for epitope mapping and can be classified into three types. One of these methods involves differential chemical modification of immune complexes, revealing the amino acids of the antigen protected by the antibody (Fiedler et al., 1998; Hochleitner et al., 1999). The structure of conformational epitopes was characterized in this way. In the epitope extraction method, the antigen is first proteolyzed and the peptides containing the epitope are extracted with an antibody, generally through immunoprecipitation. The peptides bound to the antibody are then identified through their precise mass measurement (Zhao & Chait, 1994; Macht et al., 1996; Zhao et al., 1996; Jeyarajah et al., 1998; Yu et al., 1998; Kiselar & Downard, 1999). With this approach, only linear epitopes can be characterized. In the excision epitope method, the immune complex is formed before the proteolysis and the peptides remaining bound to the antibody are mass analyzed (Suckau et al., 1990; Papac et al., 1994; Macht et al., 1996; Parker et al., 1996; Jeyarajah et al., 1998). This approach, which gives the possibility of characterizing linear epitopes, was suggested also to have the potential ability to determine discontinuous epitopes, especially when it is associated with molecular modeling (Jeyarajah et al., 1998).

In the study reported here, we used the latter approach and the 3D structure defined by Li et al. (1997) to identify and localize a new conformational epitope present within the middle part of *B. burgdorferi* OspA. It appears to be one of the first reports on the characterization of a discontinuous epitope using mass spectrometry.

Results

Chemical cleavages of native OspA

OspA that contains only one methionine located at residue 22 was cleaved with CNBr into two fragments with calculated masses of 3,139 and 25,294 Da, the methionine becoming a homoserine. As evidenced by Western blotting analysis, the larger fragment corresponding to residues Lys23–Lys257 was recognized by the mAb (Fig. 1A).

Iodosobenzoic acid treatment cleaved OspA at tryptophan 200 and generated two fragments with calculated masses of 22,246 and 6,217 Da. As above, the larger fragment was detected by Western blotting analysis (Fig. 1B). Consequently, the localization of the mAb epitope could be restricted to the region Lys23–Trp200.

Proteolytic degradation of unbound OspA

As already reported (Moroni et al., 1992), the native OspA was either poorly susceptible or totally resistant to proteolysis because of the presence of the lipid anchor. Thus, the proteolysis of OspA

Fig. 1. Chemical cleavages of the native OspA. OspA was treated with (A) CNBr and (B) iodosobenzoic acid, as described in Materials and methods. The immunoreactive generated peptides were analyzed by Western Blot. 1, Molecular weights; 2, untreated OspA; 3, treated OspA.

by different enzymes was assessed with recombinant truncated OspA since this recombinant protein was recognized by human sera and mouse mAbs as well as the native OspA (data not shown). After incubating the recombinant protein for 1 h at 37 °C in the presence of 5% (w enzyme/w OspA) of each of the following enzymes: endoproteinase Arg-C, chymotrypsin, endoproteinase Lys-C, endoproteinase Asp-N, trypsin, clostripain, and endoproteinase Glu-C, the generated peptides were analyzed by SDS-PAGE and Western blotting. OspA was partially proteolyzed by each of these enzymes. However, immunoreactive fragments were only generated by endoproteinase Lys-C and endoproteinase Glu-C treatments.

Kinetics of degradation with these two proteases showed that the protein was completely degraded with endoproteinase Lys-C after 6 h of incubation (Fig. 2A) whereas, even after 20 h of incubation with endoproteinase Glu-C, OspA was only partially degraded (Fig. 3A). Western blotting analysis revealed that the different immunoreactive generated peptides ranged from 15 to 30 kDa, whereas smaller peptides were not recognized by the mAb (Figs. 2B, 3B). These results suggest again that the epitope recognized by the mAb is conformational.

Proteolytic degradation of the affinity bound OspA

The recombinant OspA was incubated with the mAb 4C10C2 covalently coupled to Sepharose beads. The affinity bound OspA was

Fig. 2. Proteolytic degradation of the recombinant OspA with endoprotease-Lys C. OspA was incubated with endoprotease Lys-C for different periods of time as described in Results. The generated peptides were analyzed by (A) SDS-PAGE and (B) Western Blot. 1, Molecular weights; 2, untreated OspA; 3, treated OspA after 30 min of proteolysis; 4, treated OspA after 1 h of proteolysis; 5, treated OspA after 2 h of proteolysis; 6, treated OspA after 6 h of proteolysis; 7, treated OspA after 20 h of proteolysis.

then degraded with endoprotease Lys-C and endoprotease Glu-C. The supernatants, as well as the washing solutions containing the unbound peptides, were collected. The remaining peptides, still bound to the immobilized mAb, were then eluted by incubating the beads with DEA or glycine. Both mixtures of peptides were analyzed using HPLC-MS. In control experiments without OspA, we checked that the mAb 4C10C2 coupled to Sepharose beads was not degraded by endoprotease Lys-C and endoprotease Glu-C in the same conditions. For each peptide, the intensity of selected ions was extracted, and the ion intensity of bound peptide (in solution after elution)/ion intensity of unbound peptide (in supernatant) ratio was calculated for the most abundant ion. The resulting values obtained after proteolysis with either endoprotease Lys-C (Table 1) or endoprotease Glu-C (Table 2) indicated that the fragments covered 81 and 88% of the OspA sequence, respec-

tively. Using chemical cleavages of native OspA and Western blotting analysis, the N- and C-terminal regions were clearly shown not to belong to the epitope. Therefore, for each experiment, the N- and C-terminal peptides were used as references. After endoprotease Lys-C degradation (Table 1), three peptides, two of which were overlapping, were found in the region Ala142–Lys173, with a high bound/unbound ratio (45, 95, and 45 corresponding to peptides Ala142–Lys159, Glu144–Lys159, and Thr160–Lys173, respectively), compared to those of the reference peptides. These results indicated a strong interaction of the region Ala142–Lys173 with the mAb. The signals extracted from both TIC for the doubly- and triply-charged ions of peptide 144–159 are presented in Figure 4. Two other peptides (Lys196–Lys215 and Thr216–Lys223) were also found with bound/unbound ratio higher than 4 (9 and 5, respectively), indicating that the interaction of the mAb 4C10C2

Fig. 3. Proteolytic degradation of the recombinant OspA with endoprotease-GluC. OspA was incubated with endoprotease Glu-C for different periods of time as described in Results. The generated peptides were analyzed by (A) SDS-PAGE and (B) Western Blot. 1, Molecular weights; 2, untreated OspA; 3, treated OspA after 30 min of proteolysis; 4, treated OspA after 1 h of proteolysis; 5, treated OspA after 2 h of proteolysis; 6, treated OspA after 6 h of proteolysis; 7, treated OspA after 20 h of proteolysis.

Table 1. Behavior of the peptides obtained after digestion of affinity bound OspA with endoproteinase Lys-C and elution with DEA^a

M (Da)	Sequence	Charge	Bound	Unbound	B/U ratio
1,275.5	12–23	2	2,540	3,060	0.8
2,059.4	12–30	3	1,205	1,030	1.2
1,337.5	33–44	2	120	450	0.3
1,821.1	33–48	3	175	2,895	0.1
2,309.6	33–53	4	480	1,645	0.3
1,073.2	54–64	2	315	1,195	0.3
1,387.5	54–67	2	195	375	0.5
5,337.1	54–103	6	65	285	0.2
1,780.0	72–87	2	25	285	0.1
2,209.4	72–91	3	90	1,050	0.1
1,104.3	88–97	2	35	440	0.1
1,807.1	120–135	3	1,425	2,240	0.6
1,949.3	142–159	3	1,125	25	45.0
1,750.0	144–159	3	1,905	20	95.2
1,280.4	148–159	2	1,800	2,440	0.7
1,475.8	160–173	3	2,010	45	44.7
1,809.9	178–195	2	1,115	755	1.5
1,938.0	178–196	3	1,680	850	2.0
2,108.4	196–215	3	820	90	9.1
1,852.0	197–214	2	725	2,195	0.3
951.1	216–223	2	215	45	4.8
721.9	218–223	1	730	500	1.5
1,697.8	224–238	2	150	675	0.2
3,324.7	224–253	3	870	440	2.0
1,644.9	239–253	3	375	2,490	0.2

^aFor each peptide, the charge indicates the number of protons bound to the most intense ion obtained by MS; “bound” indicates the signal intensity ($\times 10^3$) of the most intense ion after elution; and “unbound” indicates the signal intensity ($\times 10^3$) of the most intense ion before elution.

with the region Lys196–Lys223 was weaker. In contrast, all the other peptides were found with ratios smaller than 2, indicating nonsignificant affinity of the mAb for the other regions of OspA. As an example of nonsignificant affinity, the signals extracted from both TIC for the doubly-, triply-, and quadruply-charged ions of peptide 33–53 are presented in Figure 5.

Using endoproteinase Glu-C (Table 2), only one peptide (Gly153–Glu166) was found with a ratio higher than 1 (3.5). This peptide was located within the region Ala142–Lys173 previously found as strongly recognized by mAb 4C10C2. However, two other peptides (Val145–Glu152 and Lys159–Glu166) had weak but significant ratios compared to those of reference peptides. This suggests that aminoacids within these two regions interact with the mAb.

Immunoanalysis of peptides

Peptides corresponding to regions Ala142–Lys159, Thr160–Lys173 were chemically synthesized and tested by ELISA. None of these peptides were recognized by the mAb 4C10C2. This confirmed the Western blotting analysis showing that proteolysis of unbound OspA did not produce any immunoreactive peptides smaller than 15 kDa (Figs. 2B, 3B). Moreover, the mAb binding to OspA was not inhibited in the presence of increasing amounts of peptides. These results suggest that within each peptide, only a few ami-

Table 2. Behavior of the peptides obtained after digestion of affinity bound OspA with endoproteinase Glu-C and elution with glycine^a

M (Da)	Sequence	Charge	Bound	Unbound	B/U ratio
1,144.3	11–21	2	15	365	0.0
933.2	22–29	2	40	290	0.1
1,950.2	30–46	3	30	420	0.1
1,518.6	47–61	2	35	235	0.1
2,446.8	62–84	3	135	290	0.5
2,038.4	89–107	3	5	245	0.0
1,687.8	116–130	3	50	125	0.4
1,372.6	119–130	3	355	1,300	0.3
1,440.6	131–144	3	20	135	0.1
920.1	145–152	2	1,130	1,420	0.8
1,489.7	153–166	3	195	55	3.5
917.1	159–166	2	910	1,235	0.7
1,420.7	167–180	2	10	690	0.0
1,835.0	167–184	3	80	175	0.5
4,274.8	185–224	6	80	220	0.4
1,810.9	225–240	2	380	1,090	0.4
685.9	252–257	1	75	470	0.2

^aFor each peptide, the charge indicates the number of protons bound to the most intense ion obtained by MS; “bound” indicates the signal intensity ($\times 10^3$) of the most intense ion after elution; and “unbound” indicates the signal intensity ($\times 10^3$) of the most intense ion before elution.

noacids interact with the mAb. After proteolysis of bound OspA, the mAb retains some affinity for the resulting bound peptides that were not washed out. However, this affinity is too weak to be detected in an ELISA test.

Localization of 4C10C2 epitope on the OspA sequence

We took into account the overlapping localization of the different fragments as well as the respective sites of cleavage of the two endoproteases that were shown to be accessible. We estimated that the Glu144–Lys159 fragment obtained after Lys-C proteolysis (B/U ratio 85 in Table 1) could be restricted to Val145–Glu152 as this fragment gave a rather high B/U ratio (0.8) compared to the reference peptides (0.0 and 0.2 for the N- and C-terminal peptides, respectively), after Glu-C proteolysis (Table 2). The Thr160–Lys173 fragment obtained after Lys-C proteolysis (B/U ratio 45 in Table 1) could also be restricted to Thr160–Glu166, taking into account the Gly153–Glu166 fragment having the highest B/U ratio (3.5), as well as the Lys159–Glu166 (ratio 0.7), after Glu-C proteolysis (Table 2).

We could thus restrict the epitope within the regions Val145–Glu152 and Thr160–Glu166 (Fig. 6).

Discussion

The interaction between antibodies and antigens represents a major aspect of the immune response. The precise characterization of epitopes is a first step in understanding the molecular mechanisms of antibody–antigen interactions. It is also useful in the design of vaccines or diagnostic tests. The term epitope mapping is currently used to describe the different methods used to determine the major

Fig. 4. Signals extracted from total ion currents for the ions of peptide 144–159. **B:** After endoproteinase Lys-C digestion of the immune complex, the supernatant was first analyzed. **A:** Following an elution with diethylamine, the washing solution was then analyzed.

Fig. 5. Signals extracted from TICs for the ions of peptide 33–53. **B:** After endoproteinase Lys-C digestion of the immune complex, the supernatant was first analyzed. **A:** Following an elution with diethylamine, the washing solution was then analyzed.

sites on a surface protein that can elicit an antibody response. The multiple synthesis of overlapping peptides corresponding to the antigen sequence that was developed by Geysen *et al.* (1985) allows to localize continuous epitopes only. If the epitope is composed of discontinuous fragments, exhaustive mutagenesis may be

undertaken for its characterization. Another methodology, the phage library technology, is a powerful tool for epitope mapping of antibodies (Scott & Smith, 1990; Jolivet-Reynaud *et al.*, 1999; Michel *et al.*, 1999). However, this is an indirect method since the selected peptides mimic the binding characteristics of the natural

Fig. 6. Structure of OspA. The regions bound to the monoclonal antibody and forming the conformational epitope are colored in dark gray.

epitope. Consequently, if the epitope is continuous, the homology between phage epitope sequences and the antigen sequence may be high, whereas in the case of noncontinuous epitopes, there may be little or no homology with the antigen sequence. Biophysical techniques such as crystallography or NMR spectroscopy may also be used for such epitopes. The crystal structure of OspA was solved recently in a complex with the Fab fragment of mouse mAb 184.1. The resolution of this structure enables the unambiguous identification of the epitope after time-consuming steps. Crystallography gives very precise information on such interactions, but the first step, the crystallogenesis, generally needs large amounts of proteins and does not always yield crystals suitable for X-ray diffraction. NMR spectroscopy was also used to characterize discontinuous epitopes of OspA. After assignment of the backbone atoms of OspA, Huang et al. (1998) identified epitopes to two mAbs (184.1 and 336) by comparing NMR chemical shifts of free OspA with those in Fab complexes. This other powerful technique, however, is still limited to antigens with a size close to that of OspA. Furthermore, NMR spectroscopy needs large amounts of concentrated proteins with good stability at rather high temperatures (above 30 °C).

In contrast, the approach we described in this study, based on mass spectrometry, takes advantage of the intrinsic features of this technique: the experiments are rather fast to undertake and need relatively small amounts of proteins.

We localized the epitope of a recombinant OspA lacking the lipidic anchor to mAb 4C10C2 to the regions Val145–Glu152 and

Thr160–Glu166. This location is consistent with our preliminary experiments using chemical cleavages and Western blotting analyses of native OspA. These experiments clearly showed that N- and C-terminal regions of OspA and, in particular, the lipid anchor bound to the N-terminal cysteine are not necessary for the recognition by our mAb. The regions we identified as forming the epitope are located in the central part of OspA, at the interface between the central sheet and barrel sheet 1. The calculated surface area of this epitope is 1,400 Å², in the same order of magnitude as the surface area constituted by residues strongly perturbed with Fab 336 (1,206 Å²) (Huang et al., 1998). Similar to the Fab 336 epitope, located at the opposite end of OspA from the membrane anchor (Huang et al., 1998), this epitope should point outward and be readily accessible on the whole spirochete. Within our epitope, only 2 residues out of 15 are strictly conserved in 49 OspA sequences (Li et al., 1997). This indicates that the mAb 4C10C2 is specific toward *B. burgdorferi* OspA American strain B31, which has been used to raise this mAb. In an ELISA test, mAb 4C10C2 also bound to the American strain G53 but not to the Swiss strain Sta3 (C. Jolivet-Reynaud & N. Battail-Poirot, pers. data). This finding is in agreement with previous biochemical and immunochemical studies, which have shown that the OspA and OspB proteins from strains of different origins may differ considerably in their reactivities with monoclonal antibodies and that this heterogeneity is more prominent among European isolates than among the North American *B. burgdorferi* isolates (Barbour et al., 1985; Wilske et al., 1988; Jonsson et al., 1992). In contrast, mAb 184.1, which was raised against sonicated spirochetes, recognizes an epitope near the N-terminus of OspA that is not readily accessible on intact spirochetes. Consistent with the broad specificity of this mAb, the epitope overlaps a highly conserved region of OspA (Li et al., 1997).

Previous studies have reported that the antibody response to OspA occurs late in the course of the Lyme disease. However, an early IgM response has been found by using different truncated constructs of recombinant OspA. This response was sometimes accompanied by a weak IgG response against epitopes located in the OspA-2 middle fragment (Kalish et al., 1995).

Our study has evidenced the presence of a conformational epitope. The major site of interaction with the antibody is located within the regions Val145–Glu152 and Thr160–Glu166 of OspA. The identification of this epitope provides new information on the immunogenicity of OspA. Furthermore, this epitope could discriminate between American and European isolates.

Materials and methods

Antigen

The native OspA was obtained from *B. burgdorferi* type strain B31 (ATCC 35210) cultures and purified according to Hansen et al. (1988). The protein was then immunopurified to homogeneity with the mouse monoclonal anti-OspA 4C10C2.

The recombinant OspA was obtained from Transgene (Strasbourg, France) as a truncated protein lacking the N-terminal lipidated cysteine (des-cys-OspA) and expressed in *Saccharomyces cerevisiae* (Mendoza-Vega et al., 1996).

Monoclonal antibody (mAb)

The mouse monoclonal anti-OspA clone 4C10C2 was obtained from BioMerieux (Marcy-l'Etoile, France). BALB/c JYco female

mice, 4 to 6 weeks old (IFFA Credo, France), were immunized by intraperitoneal injection with 15 μ g of native OspA and emulsified with an equal volume of Freund's complete adjuvant. Five injections were then made, using incomplete adjuvant every two weeks. Four days after the last injection, spleen cells were harvested and fused according to Köhler and Milstein (1975, 1976) with the Sp 2/0-Ag14 mouse myeloma cell line. After 12–14 days, the culture supernatants were screened with an ELISA test in which the solid phase was coated with the antigen used for immunization. Positive colonies were subcloned twice by limiting dilutions. Ascitic fluids were obtained from mice primed with a 0.5 mL intraperitoneal injection of Pristane and then injected with 106 hybridoma cells. IgG antibodies were purified on a protein A-Sepharose 4FF column according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden).

Enzymes

Endoproteinase Lys-C, endoproteinase Glu-C, α -chymotrypsin, proteinase K, endoproteinase Arg-C, endoproteinase Asp-N, and clostripain were obtained from Boehringer Mannheim (Mannheim, Germany). Trypsin was from Sigma (St. Louis, Missouri).

Proteolytic degradation of unbound OspA

The enzymes were diluted in appropriate buffers and added to aliquots of OspA solution at the final concentration of 5% (w/w) of OspA protein. The mixtures were then incubated at 37°C for different periods of time before analyzing the resulting fragments.

Cyanogen bromide cleavage

Chemical cleavage of OspA with cyanogen bromide (Sigma) was performed according to Gross (1967). Briefly, 100 μ L of OspA samples (10 μ g) were dissolved in 70% trifluoroacetic acid (v/v). CNBr was added to a final ratio of 5 (CNBr weight/OspA weight). The mixture was incubated for 24 h under nitrogen atmosphere, in the dark. The reaction was then stopped by adding 10 volumes of water and the solution was lyophilized.

Iodosobenzoic acid cleavage

Chemical cleavage of OspA with iodosobenzoic acid was performed according to Mahoney and Hermodson (1979). OspA samples (10 μ g) were solubilized in guanidine hydrochloride 4 M, 80% acetic acid containing 20 μ L/mL of p-cresol, and 13 mg/mL of iodosobenzoic acid. The mixture was incubated for 24 h at room temperature, in the dark.

SDS-PAGE and Western-blotting

OspA and OspA fragments were separated under reducing conditions on 12% SDS-polyacrylamide gels as described by Laemmli (1970). After separation, the proteins were transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Bedford, Massachusetts) according to Towbin et al. (1979) and blocked with 5% nonfat milk-TBS (Tris 20 mM, NaCl 500 mM pH 7.5) for 2 h at room temperature. Anti-OspA mAb dilution to the final concentration of 10 μ g/mL in TBS-milk containing 0.05% Tween20 (TBS-T-milk) were incubated with membranes, overnight, at 4°C. The membranes were then washed three times with TBS-T and

incubated with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) at a 1:5,000 dilution in TBS-T-milk (1 h, room temperature). After two washes with TBS-T and one wash with Tris-HCl 0.1 M pH 7.5, the staining was developed by incubating the membranes few minutes with substrate solution (Tris-HCl 0.1 M, pH 7.5; 0.5 mg/mL diaminobenzidine; 0.05% H₂O₂). The membranes were then washed with distilled water and dried at 37°C.

Preparation of CNBr-activated Sepharose-immobilized 4C10C2mAb

CNBr-activated Sepharose-immobilized mAb was prepared in a batch, according to the procedures provided by the manufacturer (Pharmacia Biotech, Piscataway, New Jersey). Briefly, 1 g of dry beads was washed several times with 1 mM HCl and then suspended in the coupling buffer (0.1 M NaHCO₃ pH 9.6, NaCl 0.5 M). The swollen gel (3 mL) was then incubated for 2 h, at room temperature, with 8 mg of mouse monoclonal antibody in the coupling buffer. The unreacted sites were then blocked with 0.2 M glycine, pH 8. Unbound mAb was removed with a series of washes at alternating pH 4.0 and pH 8.0.

Affinity binding of OspA to the immobilized mAb

In preliminary experiments, the optimal conditions of OspA binding as well as proteolytic degradation and elution of bound peptides were obtained with small volumes of Sepharose immobilized mAb. Thus, each experiment was performed as follows: triplicate of 200 μ L aliquots of 1.4 mg/mL OspA solution in 50 mM Tris pH 7.5, NaCl 0.15 M were added to 20 μ L of Sepharose-immobilized mAb in Eppendorf tubes and incubated for 2 h at room temperature with slow rotation. The tubes were then centrifuged and the supernatants were discarded. The beads were washed three times with 50 mM NH₄HCO₃ pH 7.8 to remove any unbound protein.

Proteolytic degradation of the affinity bound OspA

Five microliters of 1 mg/mL of enzyme solution (endoproteinase Glu-C or endoproteinase Lys-C) were added to the 20 μ L of beads containing OspA affinity-bound to 4C10C2 mAb. The mixture in a final volume of 100 μ L in 50 mM NH₄HCO₃ pH 7.8 was incubated at 37°C. After 1 or 2 h, the tubes were centrifuged, the supernatants were collected, and the beads were washed three times with the NH₄HCO₃ buffer to remove unbound cleavage products.

Elution of the bound peptides

The bound peptides were eluted by incubating the beads with 40 μ L of 0.1 M DEA, pH 11.5 or 0.1 M glycine, pH 2.8 for 1 h at room temperature with slow rotation. The mixture was then centrifuged and the supernatant was collected. Using Western blotting analyses, we checked that the peptide profile of each triplicate was similar. The supernatants corresponding to triplicate of one experiment were pooled for LC-MS analysis.

Mass spectrometry analysis

The mixtures of peptides were separated and analyzed using HPLC directly coupled with the mass spectrometer. A Carlo Erba Phenix

20 HPLC system fitted with a homemade 0.25×150 mm fused-silica column packed with $5 \mu\text{M}$ C_8 Nucleosil stationary phase was used for the chromatographic separations with a C6W Valco injector equipped with a $0.5 \mu\text{L}$ internal loop. The HPLC flow rate was $5 \mu\text{L min}^{-1}$ with a gradient of 5–60% B in 50 min (A: H_2O -formic acid 99.9:0.1 and B: acetonitrile- H_2O -formic acid 90:9.9:0.08). The mass spectrometer was a Perkin-Elmer Sciex (Foster City, California), model API III+, equipped with an electrospray source. Mass spectra were acquired during the HPLC run, with Q3 scanings in the 400–1,600 range of m/z in steps of 0.9 m/z and 2 ms dwell time.

Assays with synthetic peptides

ELISA wells were coated with $100 \mu\text{L}$ of either peptides or recombinant OspA at the concentration of $5 \mu\text{g/mL}$ as previously described (Jolivet-Reynaud et al., 1998). Competition experiments were performed by incubating $100 \mu\text{L}$ of mAb at the final concentration of 500 ng/mL with increasing amounts of peptides ranging from 1 to $25 \mu\text{g}$. After 30 min at 37°C , the mixtures were added to OspA coated wells and the followings steps were performed as before.

Molecular graphics

The representation of the structure was made with the WebLab ViewerPro software. Solvent-accessible surface areas were calculated using this software with a probe radius of $1.4 \text{ \AA}$.

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