Membrane Topology of *Borrelia burgdorferi* and *Treponema pallidum* Lipoproteins

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A critical issue regarding the molecular architectures of *Treponema pallidum* and *Borrelia burgdorferi*, the agents of venereal syphilis and Lyme disease, respectively, concerns the membrane topologies of their major lipoprotein immunogens. A related question is whether these lipid-modified membrane proteins form intramembranous particles during freeze fracture electron microscopy. To address these issues, native borrelial and treponemal lipoproteins were reconstituted into liposomes of diverse composition. The importance of the covalently associated lipids for membrane association of lipoproteins was revealed by the observation that nonlipidated recombinant forms of both *B. burgdorferi* OspA and the *T. pallidum* 47-kDa immunogen (Tpp47) showed very weak or no binding to model bilayer vesicles. In contrast to control liposomes reconstituted with bacteriorhodopsin or bovine rhodopsin, two well-characterized transmembrane proteins, none of the lipoprotein-liposomes contained particles when examined by freeze fracture electron microscopy. To extend these findings to prokaryotic lipoproteins with relatively amphiphilic polypeptides, similar experiments were conducted with a recombinant nonlipidated form of *Escherichia coli* TraT, a lipoprotein which has putative transmembrane domains. The nonlipidated TraT oligomers bound vesicles derived from *E. coli* lipids but, surprisingly, did not form particles in the freeze-fractured liposomes. These findings support (i) a proposed topology of spirochetal lipoproteins in which the polypeptide is extrinsic to the membrane surface and (ii) the contention that particles visualized in freeze-fractured spirochetal membranes represent poorly characterized transmembrane proteins.

The spirochetal bacteria *Borrelia burgdorferi* and *Treponema pallidum* are the causative agents of Lyme disease and venereal syphilis, respectively. Like all spirochetes, *B. burgdorferi* and *T. pallidum* consist of an outer membrane that surrounds the periplasmic endoflagella, the cytoplasmic membrane, and the protoplasmic cylinder (3, 32). Combined molecular and ultrastructural analyses have led to the proposal that the membrane architectures of these spirochetal pathogens differ markedly from those of enteric gram-negative bacteria (45). According to this proposal, the majority of integral membrane proteins in both spirochete species contain covalently bound lipids in a configuration identical to that of murein lipoprotein of *Escherichia coli* (29, 44), while comparatively small proportions of the total integral membrane proteins contain transmembrane domains.

Freeze fracture electron microscopy (EM) has proven a technique of choice for ultrastructural analysis of *T. pallidum* and *B. burgdorferi*. The salient feature of this method is the observation of intramembranous particles (IMPs) that are presumed to correspond to integral membrane proteins (23). Of particular importance for understanding host-pathogen relationships in both Lyme disease and syphilis, freeze fracture EM of *B. burgdorferi* and *T. pallidum* outer membranes revealed a paucity of particles relative to the typical particle-dense outer membranes of gram-negative bacteria (11, 47, 50, 61, 63, 64). This result was particularly striking for *T. pallidum*, which exhibits extremely low outer membrane particle densities (11, 50, 64).

A major premise of our proposed model for spirochetal membrane architecture is that the lipoproteins do not yield IMPs by freeze fracture EM and that the IMPs visualized in both *T. pallidum* and *B. burgdorferi* are exclusively proteins with transmembrane domains. A corollary of this premise is that freeze fracture EM provides no information regarding the relative abundance or cellular locations of the spirochetal lipoproteins. The assumption that spirochetal lipoproteins do not form IMPs derives from the general belief that only proteins with transmembrane domains form particles during the freeze fracture process (23). Although the polypeptide portions of spirochetal lipoproteins are generally hydrophilic (9, 24, 41), membrane insertion of hydrophobic domains may still occur. Alternately, interactions between the lipoproteins and neighboring lipids could induce nonspecific perturbations visualized as lipidic particles (62). Interestingly, to our knowledge, the freeze fracture EM morphology of proteins with covalent lipid anchors has never been described.

There are several reasons why it is important to clarify the freeze fracture EM behavior and membrane topologies of spirochetal lipoproteins. It cannot be stated with certainty that the low-density particles visualized in *T. pallidum* and *B. burgdorferi* outer membranes do, in fact, represent uncharacterized rare transmembrane proteins until it is proven that lipoproteins do not form particles. In the case of *B. burgdorferi*, several outer surface proteins have been proposed as vaccine candidates (20, 21, 43); a more precise understanding of the molecular organization of these proteins in their native membrane environments seems desirable. Although *T. pallidum* does not appear to have surface-exposed lipoproteins (17, 45), a detailed understanding of treponemal lipoprotein-membrane interactions could be important for elucidating the physiological functions of these molecules (6, 67). Lastly, the findings that

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spirochetal lipoproteins have proinflammatory activities *in vitro* have led to the proposal that these molecules are important inflammatory mediators during syphilis and Lyme disease (46, 49, 57). Improved understanding of the membrane topology of spirochetal lipoproteins could provide insights into how these molecules stimulate cell signaling pathways in immune effector cells.

In the present study, liposome reconstitution methodologies were used to investigate protein-lipid interactions and membrane topologies of spirochetal lipoproteins. For comparison purposes, the experiments included bacteriorhodopsin (BR) and rhodopsin (Rho), two transmembrane proteins whose physicochemical properties, membrane topologies, and freeze fracture EM behaviors have been extensively analyzed (15, 25, 27, 31, 51, 52). The major objectives of these investigations were (i) to develop a more precise understanding of how the polypeptide portions of lipoproteins interact with membrane bilayers and (ii) to relate these findings to important issues in spirochetal membrane biology and disease pathogenesis.

MATERIALS AND METHODS

Materials. All phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Octyl- β -D-glucopyranoside (OG) (reagent grade for purification and ULTRAL grade for reconstitution experiments) was purchased from CALBIOCHEM (La Jolla, Calif.). Triton X-114 and purified BR were purchased from Sigma Chemical Company (St. Louis, Mo.); the commercially obtained BR was purified by a modification of the method of Oesterhelt and Stoekenius (42). Purified bovine Rho (39) was provided by Burton J. Litman (Rockville, Md.).

Bacterial strains. *T. pallidum* (Nichols strain) was propagated intratesticularly in Venereal Disease Research Laboratory-nonreactive New Zealand White rabbits. Organisms were harvested from testicular tissue and purified by Percoll (Sigma) density gradient centrifugation (28). The avirulent (high-passage) B31 strain of *B. burgdorferi* was provided by Alan Barbour (San Antonio, Tex.) and maintained in BSKII medium (2). *B. burgdorferi* TI-1-EV was generously provided by Jorge Benach (Stony Brook, N.Y.); this strain expresses an OspAB chimera (designated OspA') in which amino acids 1 to 233 of OspA are joined by an aspartate residue to amino acids 257 to 296 of OspB (8). Glutathione S-transferase fusion proteins were expressed in *E. coli* DH5 α .

***B. burgdorferi* lipid extract.** *B. burgdorferi* lipids were isolated by solvent extraction of whole bacteria according to the procedure given by Bligh and Dyer (10).

Lipid and protein assays. Phospholipids were quantitated by P_i assay as described by Bartlett (4). Protein was assayed by either the bicinchoninic acid micromethod (Pierce Chemical Co., Rockford, Ill.) or Folin-Ciocalteu (Lowry) assay (Sigma).

Thin-layer chromatography. Commercially obtained phospholipids were examined for purity by single-dimension thin-layer chromatography on silica gel plates (9.7 cm by 3 cm by 0.25 mm; Brinkmann Instruments, Inc., Westbury, N.Y.), using a solvent system of CHCl_3 -methanol- H_2O (65:35:5); the lipids were visualized by staining with primulin. *B. burgdorferi* lipids were similarly analyzed except that staining with α -naphthol was performed to identify borrelial glycolipids.

Lipoprotein enrichment by *n*-butanol extraction. Protein mixtures enriched for OspA' and for *T. pallidum* and *B. burgdorferi* B31 lipoproteins were prepared according to the *n*-butanol extraction procedure described by Ma and Weis (40). To obtain preparations enriched for OspA' and *B. burgdorferi* B31 lipoproteins, a 500-ml stationary culture (approximately 4×10^{10} bacteria) was pelleted by centrifugation at $10,000 \times g$ for 30 min. The bacteria were washed twice with $1 \times \text{CMRL}$ and were resuspended in 8 ml of phosphate-buffered saline (PBS)-5 mM MgCl_2 . To obtain *T. pallidum* lipoprotein mixtures, approximately 3×10^{10} Percoll-purified treponemes were resuspended in 8 ml of PBS-5 mM MgCl_2 ; *n*-butanol extraction was then carried out identically on the samples as follows. Cell disruption was achieved by sonication twice for 45 s on ice (Fisher Sonic Dismembrator, Model 550, on level 5). The membrane fraction was pelleted by centrifugation at $150,000 \times g$ for 90 min and resuspended in 10 ml of cold PBS with a 30-s sonication to aid solubilization. Eight milliliters of *n*-butanol was added, and the sample was rocked gently for 1 h at 4°C. The lipoprotein-containing aqueous phase was recovered by centrifugation at $27,000 \times g$ for 90 min and dialyzed overnight against 10 mM Tris (pH 7.4)-5 mM NaCl-2 mM EDTA-1 μM pepstatin-1 μM leupeptin (chromatography starting buffer). The sample was then made 30 mM in OG.

Immunopurification of OspA'. The *n*-butanol extract from *B. burgdorferi* TI-1-EV was passed over an immunoaffinity column consisting of 1 mg each of anti-OspA monoclonal antibodies 1B11-35 and H5332 conjugated to Reacti-Gel 6X resin (Pierce). The protein was eluted with 0.25 mM acetic acid-30 mM OG; 1-ml fractions were collected into tubes containing 0.5 ml of 1 M Tris base, pH

7.8. Fractions containing purified OspA' were pooled, brought to 30 mM OG, and dialyzed against chromatography starting buffer containing 30 mM OG. The sample was then concentrated using a Centricon-10 ultrafiltration unit to a final concentration of approximately 1 $\mu\text{g}/\text{liter}$.

Production of nonlipidated proteins. A construct expressing a nonlipidated form of *E. coli* TraT was prepared by cloning a DNA fragment encoding the mature portion of this protein into the *Bam*HI and *Eco*RI sites of pGEX-2T (54). The requisite fragment was amplified by PCR from *E. coli* K-12(pRS31) (36) by using the following primers: 5'-GTCAGGATCCTGTGGTGCATGAGCA CAGC-3' (*Bam*HI site plus nucleotides 709 to 728) and 5'-CTGAGAAATCT CAGAGAATATTTGCGATTG-3' (complementary to nucleotides 1361 to 1380 plus *Eco*RI site). The sequence through the fusion joints was verified with the sequencing primer 5'-CCTTTGCAAGGCTGCAAGC-3' (nucleotides 861 to 880 of pGEX-2T). Expression of glutathione S-transferase fusions of *B. burgdorferi* B31 OspA and the 47-kDa lipoprotein of *T. pallidum* (Tpp47) was described previously (47, 66). The fusion proteins were purified by affinity chromatography on a glutathione-agarose matrix according to the manufacturer's instructions (Pharmacia, Piscataway, N.J.). To liberate the nonlipidated proteins, the fusion proteins were cleaved with thrombin before elution from the affinity matrix.

Preparation of sucrose gradients. Continuous sucrose density gradients were prepared according to the freeze-thaw procedure described by Baxter-Gabbard (5). A 10% (wt/vol) sucrose solution was subjected to one freeze-thaw cycle by freezing overnight at -20°C , followed by slow thawing in an ice water bath. Gradient formation was verified by refractive index measurements. This method produces continuous gradients of approximately 5 to 20% sucrose.

Liposome preparation. Unloaded and protein-reconstituted liposomes were prepared according to the OG dilution-dialysis procedure described by Jackson and Litman (34). All samples were prepared in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4)-100 mM NaCl-1 mM dithiothreitol-0.5 mM EDTA-0.02% NaN_3 (vesicle preparation buffer). Lipid (2.5 μmol) was initially dried by a slow nitrogen purge followed by lyophilization for at least 3 h. Solid OG, protein, concentrated buffer, and water were then added to yield an initial protein-lipid molar ratio of 1:500 at an OG concentration of 60 mM in 0.5 ml of buffer. This detergent concentration ensures complete conversion of the sample into mixed micelles (34). Samples were bath sonicated for 1 min and then incubated at 4°C for at least 4 h (for reconstitution experiments with *B. burgdorferi* or *E. coli* lipid extracts, all manipulations were carried out at room temperature to ensure that the lipids were above their gel-liquid crystalline transition temperature). The suspension was then diluted in buffer to a final OG concentration of 10 mM. This concentration is well below the critical micelle concentration of OG (22 mM) (35), which results in the conversion of micelles to vesicles as detergent is displaced to the aqueous phase. The vesicles were then dialyzed extensively against buffer to remove OG. Following dialysis, the vesicles were concentrated using Centricon-30 ultrafiltration units to about 0.5 ml and loaded onto 5 to 20% sucrose gradients prepared as described above. Vesicles were resolved by centrifugation overnight at $150,000 \times g$ at 4°C. Bands corresponding to protein-enriched vesicles were isolated from the gradient, dialyzed to remove sucrose, and concentrated for freeze fracture EM analysis by either ultracentrifugation or Centricon filtration. Aliquots of samples were retained for protein and lipid analysis. Lipid and protein gradient profiles for reconstituted vesicles were evaluated by phospholipid and immunoblotting analyses, respectively. For Rho samples, reconstitution was carried out under dim red light to prevent bleaching of the protein. Samples were then allowed to bleach prior to subsequent manipulations.

Vesicle-binding assay. Protein binding to either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles or vesicles consisting of 70 mol% POPC and 30 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) was determined by using the biotinylated DHPE-streptavidin centrifugation assay as described by Totorella et al. (59). Briefly, protein-lipid recombinant vesicles were prepared as described above with 1.6% (wt/wt) biotin-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE) added. The lipid concentration was then adjusted to 1.3 mg/ml (1.7 mM) by Centricon filtration, and streptavidin was added to give a streptavidin/biotin molar ratio of 1 to 8. Streptavidin addition results in cross-linking of vesicles, which permits pelleting under mild centrifugation conditions; unincorporated proteins remain in the supernatant. Samples were incubated at room temperature for 30 min, followed by pelleting by centrifugation at $11,000 \times g$ for 20 min. Lipid and protein in the pellet and supernatant fractions were determined by phospholipid and protein assays.

Triton X-114 phase partitioning. Analysis of protein partitioning between aqueous and detergent phases was carried out using the Triton X-114 procedure described previously (13).

Freeze fracture EM. Liposome samples were made 20% (vol/vol) with glycerol as a cryoprotectant. Approximately 2- μl portions of each sample were placed in gold sample holders designed for Balzers freeze fracture units. The holders were plunged by hand into Freon or liquid ethane cooled with liquid nitrogen and kept immersed for 5 to 6 s. The samples were then transferred rapidly to liquid nitrogen for storage prior to freeze fracture. Specimens were placed on a Balzers 400 stage cooled in liquid nitrogen. The stage with the specimens was then placed inside the Balzers unit precooled to -170°C at a vacuum of 4×10^{-2} mPa. The unit temperature was then raised to -105°C , and the specimens were fractured and coated immediately with platinum at 45°C and carbon at 90°C . The replicas were then floated in undiluted commercial bleach (Clorox) for approximately

FIG. 1. SDS-PAGE analysis of spirochetal and recombinant proteins used in this study. Samples were resolved on 12.5% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. Lanes: A, purified OspA'; B, *n*-butanol extract of *T. pallidum*; C, *n*-butanol extract of *B. burgdorferi* B31; D, nonlipidated OspA; E, nonlipidated 47-kDa *T. pallidum* immunogen; F, nonlipidated TraT.

12 h, washed three times in double-distilled water, and then transferred to copper 200-mesh grids (Energy Beam Sciences, Agawam, Mass.).

Replicas were examined and photographed with a JEOL 100SX EM at 80 kV of accelerating voltage. To ensure correct identification of fracture faces and associated structures, all specimens were photographed in stereo with 6° tilts of the goniometer; the resulting negatives were examined as stereo pairs. At least 30 fields containing from 5 to 25 liposomes per field were photographed for each sample. The sizes of the various liposome preparations as determined by freeze fracture EM were confirmed by negative staining of identical preparations, using 1% uranyl acetate.

Immunologic reagents. Rabbit polyclonal antiserum directed against *B. burgdorferi* OspA was produced as previously described (47). Human syphilitic serum was obtained from a patient with classic skin manifestations of secondary syphilis and reactive nontreponemal and treponemal tests. Antibodies against BR, Rho, and nonlipidated TraT were produced by immunizing Sprague-Dawley rats with 20 µg of purified protein in Freund's complete adjuvant, with two booster injections at 3-week intervals consisting of 10 µg of protein in Freund's incomplete adjuvant.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (38). Unless otherwise noted, samples were boiled for 5 min in final sample buffer consisting of 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with 5% (vol/vol) β-mercaptoethanol prior to electrophoresis through 2.4% stacking and 12.5% separating gels. Gels were then either stained in Coomassie brilliant blue or transferred electrophoretically to 0.2 µm-pore-size nitrocellulose for immunoblotting.

Binding of dig-amp by liposome-reconstituted OspA' and Tpp47. Digoxigenin-ampicillin conjugate (dig-amp) was prepared as previously described by Weigel et al. (65). Liposomes (10-µl volumes) containing approximately 50 ng of reconstituted OspA' or an *n*-butanol extract of *T. pallidum* were incubated with dig-amp (10 µg/ml) for 10 min at 34°C. The samples were then boiled in final sample buffer, separated on 12.5% SDS-polyacrylamide gels, and processed for chemiluminescence (65).

RESULTS

Protein analysis. The spirochetal and recombinant protein preparations used in this study are shown in Fig. 1. Inasmuch as OspA and OspB are not easily separated under nondenaturing conditions (12), a *B. burgdorferi* strain expressing a single OspAB chimera (designated OspA') composed predominantly of OspA was used for experiments requiring a purified borrelial lipoprotein (Fig. 1, lane A). Because of the difficulty in obtaining purified individual *T. pallidum* lipoproteins in amounts necessary for reconstitution experiments, lipoprotein-enriched preparations were obtained by *n*-butanol extraction of whole *T. pallidum*; the treponemal polypeptides visualized by SDS-PAGE (Fig. 1, lane B) previously have been shown to

be predominantly lipoproteins (14, 48). Immunoblot analysis confirmed that the butanol-extracted material obtained from *T. pallidum* also contained the highly immunogenic 15- and 17-kDa lipoproteins (Fig. 2E). The *n*-butanol extract obtained from *B. burgdorferi* B31 was predominantly approximately equal portions of OspA and OspB (Fig. 1, lane C). The purities of recombinant nonlipidated forms of OspA, Tpp47, and TraT are shown in Fig. 1, lanes D through F, respectively; the 54-kDa band above the 27-kDa TraT monomer was confirmed to be TraT (presumably a dimer) by immunoblot analysis with a monospecific rat antiserum (data not shown).

Fractionation of protein-reconstituted vesicles. The OG dilution-dialysis method typically results in liposomes with heterogeneous protein/lipid ratios (34). To obtain a homogeneous population of vesicles with known protein/lipid ratios, vesicles were fractionated on sucrose gradients. Figure 2 illustrates the gradient profiles for unloaded POPC vesicles and POPC vesicles reconstituted with BR, Rho, OspA', and *T. pallidum* lipoprotein-enriched mixtures. Unloaded POPC liposomes formed a single peak near the top of the gradient (Fig. 2A). By contrast, the protein-POPC profiles were complex; the multiple peaks indicated that vesicles of various densities (and thus various protein/lipid ratios) were resolved by the gradient centrifugation procedure (34). For each liposome-protein preparation, immunoblot and phospholipid assays of individual gradient fractions confirmed that the proteins and lipids comigrated and, therefore, that the proteins were vesicle bound (Fig. 2). Interestingly, the immunoblot of the Rho-POPC vesicles suggested that the membrane-associated protein included aggregates (Fig. 2C). Also apparent was that migration of vesicles in the gradient depended upon the protein/lipid ratio rather than the absolute amount of protein in a given fraction (Fig. 2B to E). In additional experiments, the same proteins were reconstituted into liposomes containing POPC-POPG (70:30 mol%) (PCPG). Fractionation of the PCPG liposomes and immunoblot analysis confirmed (as before) that the proteins were vesicle bound, although the PCPG-protein profiles were less complex than those obtained for POPC-protein liposomes (data not shown).

We recently demonstrated, using a dig-amp conjugate (65), that native Tpp47 is a penicillin-binding protein and that the ability of the native protein to bind dig-amp was lost upon denaturation (67). Consistent with the report by Urban et al. (60) that OspA binds penicillin, OspA and OspA' in *B. burgdorferi* whole cells also bound dig-amp (68). POPC- and PCPG-reconstituted OspA' and Tpp47 covalently bound dig-amp, providing evidence that significant structural alterations of the spirochetal lipoproteins did not occur during reconstitution (data not shown).

Association of nonlipidated recombinant proteins with liposomes. Having established that the spirochetal lipoproteins could be reconstituted into liposomes, we next used a centrifugation-based binding assay (59) to determine whether the polypeptide portions of the lipoproteins contribute to integration into POPC or PCPG model membranes. Experiments were performed with both neutral and net negatively charged membranes to enable us to distinguish hydrophobic and electrostatic contributions to membrane binding. As shown in Fig. 3, 70% and greater than 90% of OspA' bound to POPC and PCPG vesicles, respectively. In contrast, nonlipidated OspA showed negligible binding to POPC vesicles (Fig. 3A) and only weak binding to PCPG (Fig. 3B), while the nonlipidated form of Tpp47 did not bind to either of the systems studied (Fig. 3). Essentially all of the phospholipid was pelleted in the presence or absence of protein (Fig. 3).

FIG. 2. Sucrose density gradients of POPC liposomes (A) and POPC liposomes reconstituted with BR (B), Rho (C), OspA' (D), and *n*-butanol extract of *T. pallidum* (E). The solid curves represent the phospholipid profiles, while the dashed curves are the density gradients. The corresponding immunoblots of the gradient fractions are shown above the gradient for each protein-loaded sample. The vertical lines denote the fractions that were combined and used in subsequent freeze fracture EM experiments.

Freeze fracture EM analysis of vesicles. Freeze fracture EM was performed to explicitly address the issue of particle formation by liposome-reconstituted lipoproteins. Initially, POPC liposomes were studied. Unloaded liposomes had smooth fracture faces, as expected (Fig. 4A). Compared with the unloaded liposomes, Rho-POPC and BR-POPC vesicles were considerably larger (mostly 80 to 200 nm in diameter as opposed to 20 to 40 nm for the unloaded vesicles) and exhibited numerous IMPs distributed uniformly over the convex and concave fracture faces (Fig. 4B and C). Rho-POPC showed particles ranging from approximately 5 to 20 nm in diameter at an average density of $1,400/\mu\text{m}^2$. BR-POPC yielded particles ranging from 5 to 15 nm at densities from $1,000$ to $1,500/\mu\text{m}^2$. It is also noteworthy that in the various lipid systems, Rho formed more distinct particles than did BR; this difference was most pronounced in the POPC and PCPG liposomes (Fig. 4B and C and 5B and C, respectively). For both the Rho- and BR-POPC

systems, greater than 90% of the vesicles had particle densities that fell within 30% of the average density value. For both BR and Rho, smooth vesicles and extremely particle-laden vesicles also were occasionally observed (Fig. 4C and not shown, respectively). In contrast to both the Rho- and BR-reconstituted liposomes, IMPs were not evident in POPC liposomes reconstituted with either an OspA'- or a *T. pallidum*-lipoprotein mixture (Fig. 4D and E). The lipoprotein-reconstituted liposomes also were comparatively small (about 20 nm in diameter).

To rule out the possibility that the lack of IMPs in lipoprotein-reconstituted liposomes was a peculiarity of the POPC system, freeze fracture EM was performed on liposomes reconstituted with lipid mixtures which more closely resembled native spirochetal lipids (7). As shown in Fig. 5, the freeze fracture EM morphologies of the PCPG and PCPG-protein liposomes were essentially identical to their POPC counterparts. As a further confirmation that the lipid environment did not influence particle formation by lipoproteins, OspA' was reconstituted into a borrelial lipid extract consisting largely of PC and PG but also containing several poorly defined glyco-

FIG. 3. Binding of OspA', nonlipidated OspA, and nonlipidated Tpp47 to POPC (A) and POPC-POPG (70:30 mol%) (B) liposomes.

lipids (7). Density gradient analysis of borrelial lipids reconstituted with OspA', Rho, and BR confirmed that all three proteins were vesicle associated, and thin-layer chromatography analysis showed that the lipid composition of the reconstituted vesicles was identical to that of the input lipids (data not shown). IMPs once again were clearly discernible in BR and Rho *B. burgdorferi* lipid liposomes (Fig. 6B and C), although the particles were generally larger and showed lower densities than those obtained with the model systems, particularly for Rho. Rho showed particles of mostly 10 to 20 nm with some up to 30 nm at densities of $650/\mu\text{m}^2$, while BR showed mostly 10- to 20-nm particles at densities of about $1,000/\mu\text{m}^2$. In contrast, liposomes reconstituted with OspA' were smooth (Fig. 6D). Inasmuch as the freeze fracture EM results for OspA' did not rule out the possibility that other borrelial lipoproteins could form IMPs, a *B. burgdorferi* protein preparation which consisted predominantly of approximately equivalent amounts of OspA and OspB (Fig. 1, lane C) was reconstituted with borrelial lipids. This sample also yielded smooth fracture faces (results not shown).

Membrane interactions of nonlipidated TraT oligomers. The experiments described above indicated that the polypeptide portions of spirochetal lipoproteins have little or no amphiphilic character and, thus, do not integrate into membrane bilayers. Nevertheless, it was still possible that either or both of these spirochetes possessed uncharacterized lipoproteins with relatively amphiphilic polypeptides. Because this could not be investigated directly, experiments were performed with a recombinant nonlipidated form of the surface-exposed *E. coli* TraT lipoprotein; this polypeptide contains two hydrophobic stretches, one of which is large enough to span the *E. coli* outer membrane (44, 55, 56). When we attempted to carry out the centrifugation-based liposome-binding assay for this protein, the protein pelleted in the absence of lipids under the relatively mild centrifugation conditions used for this assay. Triton X-114 phase partitioning was used, therefore, as an alternative method to examine whether the TraT polypeptide possesses hydrophobic character. While OspA' and nonlipidated OspA partitioned exclusively into the respective detergent-enriched

and aqueous phases, approximately 65% of the nonlipidated TraT partitioned in the detergent-enriched phase (data not shown). The TraT protein then was reconstituted into liposomes derived from native *E. coli* lipids (Fig. 7). As shown in Fig. 8, unloaded vesicles and TraT-reconstituted vesicles were essentially smooth, while BR- and Rho-reconstituted liposomes showed numerous IMPs of morphologies similar to those observed for the borrelial lipid extract. Inasmuch as native TraT exists as large aggregates (55), it was possible that the lack of particles was due to failure of the recombinant protein to oligomerize. For this reason, the nonlipidated TraT was examined by SDS-PAGE both without and with boiling in SDS. As reported previously for native TraT (58), the large majority of the unboiled material formed large aggregates which did not enter the separating gel (Fig. 9).

DISCUSSION

In this study, model membrane systems, including native spirochetal lipids, were used to investigate protein-lipid interactions and membrane topologies of *B. burgdorferi* and *T. pallidum* lipoproteins. At the outset, we showed that spirochetal lipoproteins could be reconstituted successfully into liposomes with protein/lipid ratios comparable to those of the positive control transmembrane proteins BR and Rho. We then went on to clarify the potential contribution of the polypeptide portions of these proteins to lipid binding and the topologic relationship(s) between the polypeptide moieties and the membrane surface. Taken as a whole, these studies showed that the polypeptide portions of the spirochetal lipoproteins contribute little to membrane binding and that the polypeptides are extrinsic to the lipid bilayer. Somewhat surprising was the observation that TraT, an *E. coli* lipoprotein whose polypeptide has significant amphiphilic character, also did not form particles in freeze-fractured liposomes. Findings for the lipoproteins contrasted sharply with those obtained for the well-characterized transmembrane proteins BR and Rho, both of which integrated into membranes of diverse composition and formed discrete particles when examined by freeze fracture EM.

The membrane affinities of the polypeptides were determined by assaying binding of nonlipidated forms of OspA and Tpp47 to model bilayer vesicles. Both recombinant proteins failed to bind to neutral POPC vesicles even at the relatively high lipid concentration (about 1.7 mM) used for this experiment. These results demonstrated that there is no significant hydrophobic free energy driving association of these proteins with the apolar lipid interior. The relatively weak binding shown by the highly basic nonlipidated OspA (9) to PCPG vesicles under these conditions is typical of the surface electrostatic interaction of basic proteins with net negatively charged bilayers (37). It was also noteworthy that OspA' exhibited more complete binding to the negatively charged PCPG liposomes. The strong tendency of the hydrophobic lipoprotein to self-associate in solution most likely was overcome by electrostatic attraction between the basic OspA' polypeptide and the negatively charged lipids.

Freeze fracture EM provided additional insight into the membrane interactions of the polypeptide portions of the spirochetal lipoproteins. For these experiments, we used the well-characterized transmembrane proteins BR and Rho as positive controls. It was felt that the use of two positive controls was desirable given that a major objective of this study was to prove a negative result (i.e., the lack of particle formation by lipoproteins).

The freeze fracture EM findings for Rho and BR agreed well with both theoretical predictions and previously published

FIG. 4. Freeze fracture EM of POPC liposomes (A) and POPC liposomes reconstituted with BR (B), Rho (C), OspA' (D), and *n*-butanol extract of *T. pallidum* (E). Protein/lipid molar ratios were 1:400 for BR and Rho, 1:200 for OspA', and 1:300 for the *T. pallidum* extract. $\smile$ and $\frown$ represent the concave and convex membrane leaflets, respectively. For the purpose of clarity, relatively large unloaded and lipoprotein-loaded vesicles are shown in panels A, D, and E. Bars = 0.25 μm .

observations for these proteins following reconstitution into lipids with heterogeneous and/or *cis*-unsaturated acyl chains (15, 18, 25, 27, 33, 34, 51). The cross-sectional diameter of a single Rho molecule has been estimated as approximately 3.7 nm by a crystallographic study of Rho reconstituted in lipid planar membranes (52). Assuming that carbon-platinum shadowing adds about 1.5 to 2.0 nm to observed particle diameters (16), the 5-nm particles are within the size range predicted for Rho monomers, while the larger particles (10 to 20 nm) are likely to be small aggregates. Estimation of the anticipated particle size for a BR monomer is more problematic than for Rho inasmuch as BR is more elongated (2.5 by 3.5 by 4.5 nm) (27, 30, 31). However, once again the smaller particles almost certainly represent BR monomers and the larger particles reflect dimers and/or trimers. That the observed particle densities for both BR and Rho were approximately two- to threefold

less than the theoretical particle densities ($3,968/\mu\text{m}^2$ for each, calculated using the measured protein/lipid ratio 1/400 and a lipid cross-sectional area of 0.63 nm^2 [16]) also is consistent with the particles being a mixture of monomers and small aggregates. Compared with POPC and PCPG model systems, Rho and BR vesicles prepared with the *B. burgdorferi* or *E. coli* lipid extracts formed larger particles at lower densities. These results suggest that protein aggregation was more pronounced in the more heterogeneous lipid environments (7). It is also noteworthy that the Rho particles were generally more distinct than those formed by BR. While BR and Rho share the seven-transmembrane-helix bundle structure, the helix-connecting regions are much smaller for BR (e.g., molecular masses of 27 kDa for BR and 40 kDa for Rho) (30, 31, 52). Thus, BR is anticipated to yield particles of reduced height relative to Rho.

IMPs were not observed upon fracture of liposomes recon-

FIG. 5. Freeze fracture EM of POPC-POPG (70:30 mol%) liposomes (A) and liposomes reconstituted with BR (B), Rho (C), OspA' (D), and *n*-butanol extract of *T. pallidum* (E). Protein/lipid molar ratios were 1:350 for BR, 1:400 for Rho, 1:500 for OspA', and 1:500 for the *T. pallidum* extract. $\smile$ and $\frown$ represent the concave and convex leaflets, respectively. For the purpose of clarity, relatively large vesicles are shown in panels A, D, and E; however, note the many extremely small vesicles in panel E. Bars = 0.50 μ m.

stituted with purified OspA', mixtures of OspA and OspB, or a mixture consisting of the major *T. pallidum* lipoproteins. Moreover, this observation was consistent for the various lipid systems studied, including liposomes reconstituted with native borrelial lipids, ruling out the possibility that particle formation by spirochetal lipoproteins required a particular lipid environment. Despite the fact that none of the lipoproteins examined in this study formed particles, it was still possible that these bacteria contain other uncharacterized lipoproteins which do form IMPs. TraT, a surface-exposed *E. coli* lipoprotein with membrane-spanning domains (55), was used as a model for such a lipoprotein. Consistent with prior reports and sequence analysis (55), we confirmed the distinctive hydrophobic character of the TraT polypeptide both by reconstitution into liposomes and by Triton X-114 phase partitioning. Nevertheless, the membrane-associated oligomeric polypeptide

did not form IMPs, further supporting the generalization regarding the lack of particle formation by spirochetal lipoproteins. One potential explanation for this freeze fracture EM result is that the large majority of the TraT polypeptide exists on one side of the bilayer with relatively small hydrophobic domains "looping" into the membrane interior. Not only do these intriguing results support the conjecture by Sukupolvi and O'Connor (55) that the molecular organization of TraT within the membrane differs radically from that of porins, they appear to represent the first example of a protein possessing transmembrane domains without forming IMPs. The combined results for the vesicle binding and freeze fracture EM experiments strongly support a topology for the spirochetal lipoproteins whereby the lipid-anchored polypeptides remain extrinsic to the bilayer surface with virtually no potential to insert into the apolar membrane interior. In this regard, it was

FIG. 6. Freeze fracture EM of liposomes prepared from *B. burgdorferi* lipids (A) and liposomes reconstituted with BR (B), Rho (C), and OspA' (D). Protein/phospholipid molar ratios were 1:250 for BR, 1:350 for Rho, and 1:100 for OspA'. $\smile$ and $\frown$ represent the concave and convex leaflets, respectively. Bars = 0.50 μ m.

noteworthy that the lipoprotein-containing POPC and PCPG vesicles were generally smaller than those reconstituted with either BR or Rho. If the protein moiety of the lipoproteins does not interact with the bilayer, the proteins would serve as large "headgroups" which would stabilize smaller vesicles (26).

The topology described above has important implications for our understanding of the relationship between spirochetal molecular architecture and disease immunopathogenesis. First, failure to observe particle formation by lipoproteins in these model systems empirically verified the conjecture that IMPs in freeze-fractured *T. pallidum* and *B. burgdorferi* mem-

branes represent proteins with transmembrane domains (45). Second, the fact that the polypeptide regions of the spirochetal lipoproteins do not insert into the membrane interior implies that interactions with membrane lipids contribute little to protein folding. These results, therefore, reinforce the validity of using recombinant, nonlipidated forms of these proteins, which can be produced in relatively large quantities, to probe the biochemical, physiological, and immunological properties of the native lipoproteins (1, 19, 20, 67). Third, it has been suggested that the failure of certain monoclonal antibodies directed against OspA and OspB to passively protect animals is due to insertion of their target epitopes into the *B. burgdorferi* outer membrane (22, 53). Our data suggest that epitope inaccessibility is more likely to result from protein folding than from steric hindrance by the membrane.

Lastly, this topological arrangement raises intriguing issues regarding localization of outer surface proteins in *B. burgdorferi*. For a lipoprotein with a hydrophilic polypeptide to be surface exposed, the polypeptide must completely translocate across the outer membrane. This is in contrast to outer membrane proteins in enteric gram-negative bacteria (e.g., *E. coli* OmpA), which insert spontaneously into the outer membrane following export across the cytoplasmic membrane (44). Because the spontaneous membrane translocation rates of highly polar polypeptides are vanishingly low, secretory machinery must exist for translocating lipoproteins across the outer membrane to their final destination on the spirochetal surface. Such *sec* gene-dependent machinery has been characterized for the secretion of the lipoprotein pullulanase, a starch-debranching enzyme of *Klebsiella* species (44). Inasmuch as components of

FIG. 7. Sucrose density gradient (phospholipid) profiles of liposomes derived from *E. coli* lipids (---) and *E. coli* lipids reconstituted with nonlipidated, recombinant TraT (—). The dashed curve represents the density gradient. Immunoblot analysis of the gradient fractions with anti-TraT antiserum is shown above.

FIG. 8. Freeze fracture EM of liposomes prepared from *E. coli* lipids (A) and *E. coli* lipids reconstituted with BR (B), Rho (C), and nonlipidated TraT (D). Protein/lipid molar ratios were 1:400 for BR, 1:350 for Rho, and 1:200 for TraT. ◡ and ◢ represent the concave and convex leaflets, respectively. Bars = 0.50 μ m.

FIG. 9. Oligomeric structure of recombinant TraT. SDS-PAGE of purified, recombinant nonlipidated TraT with (lane 1) or without (lane 2) boiling in final sample buffer. The arrow indicates oligomeric TraT at the interface between the stacking and separating gels.

this secretory machinery appear to be highly conserved (44), it is tempting to speculate that a related mechanism underlies lipoprotein export in *B. burgdorferi*. The absence of surface-exposed lipoproteins in *T. pallidum* (17, 45) suggests that the syphilis spirochete does not possess comparable secretory machinery.

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