JOURNAL OF BACTERIOLOGY, Sept. 2005, p. 6499–6508 Vol. 187, No. 18
0021-9193/05/$08.00+0 doi:10.1128/JB.187.18.6499–6508.2005

TP0453, a Concealed Outer Membrane Protein of *Treponema pallidum*, Enhances Membrane Permeability

Karsten R. O. Hazlett,[1]* David L. Cox,[4] Marc Decaffmeyer,[6] Michael P. Bennett,[5] Daniel C. Desrosiers,[1] Carson J. La Vake,[1] Morgan E. La Vake,[1] Kenneth W. Bourell,[1] Esther J. Robinson,[7]† Robert Brasseur,[6] and Justin D. Radolf[1,2,3]

Center for Microbial Pathogenesis[1] and Departments of Medicine[2] and Genetics and Developmental Biology,[3] University of Connecticut Health Center, Farmington, Connecticut 06030; Division of STD Laboratory Research, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[4]; Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269[5]; Centre de Biophysique Moléculaire Numérique FSAGX, 5030 Gembloux, Belgium[6]; and Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390[7]

Received 20 April 2005/Accepted 5 July 2005

The outer membrane of *Treponema pallidum*, the noncultivable agent of venereal syphilis, contains a paucity of protein(s) which has yet to be definitively identified. In contrast, the outer membranes of gram-negative bacteria contain abundant immunogenic membrane-spanning β-barrel proteins mainly involved in nutrient transport. The absence of orthologs of gram-negative porins and outer membrane nutrient-specific transporters in the *T. pallidum* genome predicts that nutrient transport across the outer membrane must differ fundamentally in *T. pallidum* and gram-negative bacteria. Here we describe a *T. pallidum* outer membrane protein (TP0453) that, in contrast to all integral outer membrane proteins of known structure, lacks extensive β-sheet structure and does not traverse the outer membrane to become surface exposed. TP0453 is a lipoprotein with an amphiphilic polypeptide containing multiple membrane-inserting, amphipathic α-helices. Insertion of the recombinant, nonlipidated protein into artificial membranes results in bilayer destabilization and enhanced permeability. Our findings lead us to hypothesize that TP0453 is a novel type of bacterial outer membrane protein which may render the *T. pallidum* outer membrane permeable to nutrients while remaining inaccessible to antibody.

Venereal syphilis is a multistage inflammatory disease caused by the extracellular, slow-growing bacterium *Treponema pallidum*, an obligate pathogen whose ability to evade the immune response allows for persistent, even lifelong, infection (28). Because of their common double-membrane ultrastructures, spirochetes, such as *T. pallidum*, have often been analogized to enteric gram-negative bacteria, such as *Escherichia coli*. The *T. pallidum* outer membrane (OM), however, differs significantly from gram-negative OMs; these differences are thought to contribute to the bacterium's immunoevasiveness (37, 38, 46a). *T. pallidum* lacks lipopolysaccharide, the main permeability barrier of gram-negative OMs, which is a potent proinflammatory agonist and a target of serotype-specific antibodies (13). While the *T. pallidum* OM contains abundant, as-yet uncharacterized glycolipids, they are poorly antigenic (39). In addition, the host-pathogen interface of gram-negative bacteria contains a number of proteinaceous entities such as pili, flagella, and integral OM proteins (OMPs), all of which can be targets for opsonizing and/or neutralizing antibodies (35). In contrast, *T. pallidum* lacks pili, its flagella are entirely periplasmic, and its OM contains a modicum of protein(s) (38, 46a). Freeze fracture electron microscopy analysis indicates that the density of OM intramembranous particles in *T. pallidum*, presumed to represent surface-exposed, transmembrane (TM) proteins, is two orders of magnitude lower than that in *E. coli* (36).

Despite extensive efforts and a variety of experimental approaches over the past ~25 years, unambiguous identification of a *T. pallidum* OMP has eluded syphilis researchers. *T. pallidum* cannot be cultured in vitro; instead, the organism is harvested from infected rabbit testes. Existing methods for the removal of contaminating host proteins frequently damage the bacterium's exceedingly fragile OM; an underappreciation of the fragility of the *T. pallidum* OM has led to the misidentification of periplasmic inner membrane (IM) and, in some cases, cytoplasmic constituents as OMPs (36, 37). The inability to culture *T. pallidum* also has hindered the development of systems for mutagenesis of this bacterium. Consequently, genetic approaches for the identification and characterization of OMPs cannot be applied to *T. pallidum* as they have in other pathogens. The availability of the spirochete's genomic sequence (13), a watershed that has accelerated many facets of syphilis research, has provided limited evidence for recognizable OMPs. Perhaps most striking, given *T. pallidum*'s severely limited biosynthetic capacity and absolute dependence on host-supplied nutrients, is the absence of orthologs for recognizable porins and OM nutrient-specific transporters (13, 36).

* Corresponding author. Mailing address: Center for Microbial Pathogenesis, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. Phone: (860) 679-8391. Fax: (860) 679-1358. E-mail: KHazlett@up.uchc.edu.
† Retired.

In gram-negative bacteria, transport of nutrients across the OM is mediated by both general porins (OmpF, OmpC, PhoE), which have lesser degrees of selectivity, and nutrient-specific transporters (LamB, ScrY, FadL, BtuB, FhuA) (3, 22, 35). Like all integral OMPs of known structure, these proteins do not contain TM α-helices but instead are comprised of membrane-spanning β-barrels, each of which surrounds an aqueous channel that acts as a conduit for the passage of solutes (22, 35). The absence of orthologs of gram-negative porins and outer membrane nutrient-specific transporters in the *T. pallidum* genome predicts that passage of nutrients across the OM must differ fundamentally in *T. pallidum* and gram-negative bacteria. Moreover, the lack of these orthologs may relate to the bacterium's exceedingly slow doubling time, estimated to be 30 to 33 h in vivo (29).

Here we used a battery of genetic, biological, and biophysical approaches to show that TP0453 is an integral OMP with characteristics that are, to our knowledge, unprecedented among bacterial OMPs of known structure. TP0453 lacks extensive β-sheet structure and does not traverse the OM to become surface exposed. Lipid modification provides an OM tether for this partially amphiphilic polypeptide that contains multiple membrane-inserting, amphipathic α-helices. Insertion of the recombinant, nonlipidated protein into model membranes results in bilayer destabilization and enhanced permeability. Cumulatively, our findings allow us to hypothesize that TP0453 may render the *T. pallidum* OM permeable to nutrients without compromising the spirochete's low surface antigenicity.

MATERIALS AND METHODS

Bacteria. Animal protocols described in this work were approved by the University of Connecticut Health Center Animal Care Committee under the auspices of Animal Welfare Assurance number A3471-01. *T. pallidum* was propagated in rabbits as described previously (16). *E. coli* strain TOP10F′ (Invitrogen, Carlsbad, CA) was used for DNA cloning; strain BL21(DE3)-Star (Invitrogen) was used for protein expression.

[^{125}I]TID labeling. A total of 5×10^9 freshly extracted *T. pallidum* cells were harvested by centrifugation (20,000 × *g* for 20 min), resuspended very gently in 330 ml of phosphate-buffered saline (PBS) containing 5 mM $MgCl_2$, and placed in a well of a glass spot plate. A total of 100 mCi of 3-(trifluoromethyl-)-3-(*m*-[^{125}I]iodophenyl-diazarene) ([^{125}I]TID; Amersham Pharmacia Biotech, Piscataway, NJ) was added under safe-light conditions. Following a 10-min incubation, the reagent was photoactivated by exposure to long-wave UV light (366 nm) for 30 min. Following Triton X-114 phase partitioning (see below), fractions from 5×10^7 *T. pallidum* cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Identification of the gene encoding TP0453. Triton X-114 detergent-phase proteins from [^{125}I]TID-labeled *T. pallidum* were resolved by two-dimensional gel electrophoresis as described previously (1); the radioiodinated spot was excised and digested with trypsin. Sequencing of nine tryptic peptides, performed by the Howard Hughes Medical Institute Biopolymer Laboratory (University of Texas Southwestern Medical Center), enabled BLAST searches of the *T. pallidum* genomic sequence (13).

Proteins. Two constructs were generated for the expression of recombinant TP0453 (rTP0453) for biophysical characterization. The first, pET4341:TP0453, encodes a glutathione *S*-transferase-hexahistidine (H6) chimera with a thrombin cleavage site fused to the N terminus of mature, nonlipidated TP0453. pET4341, the parental vector for pET4341:TP0453, was constructed by cloning the NdeI/SpeI, glutathione *S*-transferase-encoding DNA from pET41a (Novagen, Madison, WI) into the same sites of pET43.1a (Novagen). TP0453 DNA encoding residues 27 through 287 was PCR amplified from *T. pallidum* DNA with primers TP453-5′SmaI (5′-TCCCCCGGGGTTCAGGGGCGTGGAAGGCATC-3′) and TP453-3′HindIII (5′-AGAAAGCTTTTACGAACTTCCCTTTTTGGAGTAC-3′), cloned into the SmaI/HindIII sites of pET4341, and sequenced. Frozen pellets of isopropyl-β-D-thiogalactopyranoside-induced cells were lysed in the presence of 1% *n*-octyl-β-D-glucopyranoside (OG). Soluble GSTH6TP0453 protein was purified by sequential nickel-nitrilotriacetic acid (Ni-NTA; Bio-Rad Laboratories, Hercules, CA) and glutathione-agarose (Sigma, St. Louis, MO) affinity chromatographies with buffers containing 1% Tween-20. Thrombin (Amersham)-cleaved rTP0453 was subsequently loaded onto a cation exchange column (Bio-Rad), washed extensively with 25 mM Tris (pH 7.0), 10% glycerol, 1% OG, and eluted with a 0 to 0.5 M NaCl gradient in the same buffer. Fractions containing rTP0453 were dialyzed against 25 mM Tris (pH 7.0), 150 mM NaCl, 2% OG, 50% glycerol and stored at −80°C.

The second construct, TP0453:MBPH6, encodes a TP0453 maltose-binding protein (MBP)-H6 fusion, which, due to the solubility of MBP (40), was fully soluble in the absence of detergents. TP0453 DNA encoding residues 26 through 287 was PCR amplified with primers TP0453-5′NdeI (5′-GCCATATGACTTCAGGGGCGTGGAAGGC-3′) and TP0453-3′EcoRI (5′-CGGAATTCGAACTTCCCTTTTTGGAGTACAACG-3′), cloned into the NdeI and EcoRI sites of p23bMBPH6 (40), and sequenced. Soluble rTP0453MBPH6 was purified from isopropyl-β-D-thiogalactopyranoside-induced cell lysates by Ni-NTA-chromatography with detergent-free buffers, brought to 0.83 M $(NH_4)_2SO_4$, and further purified by sequential hydrophobic interaction and anion-exchange chromatographies. The best fractions were concentrated by ultrafiltration prior to size-exclusion chromatography (Superdex 200; Amersham) using 25 mM Tris (pH 7.3), 150 mM NaCl as the mobile phase.

A control protein, MBPH6, was expressed from pET23bMBPH6 and purified by Ni-NTA and anion-exchange chromatographies, dialyzed against 25 mM Tris (pH 7.3), 150 mM NaCl, and concentrated by ultrafiltration. A nonlipidated form of the 47-kDa *T. pallidum* lipoprotein was expressed from the plasmid pProExTp47 provided by Kayla E. Hagman (University of Texas Southwestern Medical Center). Soluble H6-Tp47 was purified from isopropyl-β-D-thiogalactopyranoside-induced cell lysates by Ni-NTA chromatography and dialyzed against 25 mM Tris (pH 7.0), 150 mM NaCl, 50% glycerol prior to storage at −80°C. The *Neisseria gonorrhoeae* porin P1A was generously provided by ImClone Inc. (New York, N.Y.). The *Halobacterium salinarium* membrane protein bacteriorhodopsin (BR) (Sigma) was resuspended in 25 mM Tris (pH 7.0), 150 mM NaCl, 2% OG, 50% glycerol. Protein concentrations were determined spectrophotometrically using extinction coefficients calculated by ProtParam (www.expasy.org/tools/protparam.html).

Antibodies. TP0453 DNA was PCR amplified from *T. pallidum* genomic DNA with primers TP0453-5′DSEcoRI (5′-GCGGAATTCACTTCAGGGGCGTGGAAGGC-3′) and TP0453-3′DSBamHI (5′-GCGGGATCCTTACGAACTTCCCTTTTTGGAG-3′) and cloned into the EcoRI/BamHI sites of pMAL-c2x (New England BioLabs Inc., Beverly, MA), generating an MBP-TP0453 gene fusion. Recombinant MBP-TP0453 (rMBP-TP0453) was purified using amylose resin and cleaved with factor Xa according to the manufacturer's instructions. The identity of the immunogen was confirmed by automated Edman degradation. Rat and rabbit α-TP0453 antisera were generated as described previously (16). BR was similarly used to generate polyclonal rat α-BR antisera. A monoclonal antibody directed against *E. coli* alkaline phosphatase was purchased from Caltag Laboratories (Burlingame, CA). Polyclonal antisera directed against Tp47 and FlaA have been described already (11, 16, 42).

Triton X-114 phase partitioning. *T. pallidum* was phase partitioned as previously described (5), resolved by SDS-PAGE, and processed for silver staining, autoradiography, or immunoblotting. Triton X-114 (2% final concentration; Sigma) was added to a protein mixture containing equal amounts of rTP0453, BR, and rTp47 and dialyzed overnight against PBS at 4°C to remove OG. The proteins were partitioned into aqueous and detergent phases and washed once prior to SDS-PAGE.

Proteinase K digestion. To minimize damage to *T. pallidum* OMs, digestions were preformed on freshly extracted *T. pallidum* cells while suspended in extraction media. Following the addition of $CaCl_2$ to 1 mM and either PBS or proteinase K (Fischer Scientific, Pittsburgh, PA) to 0.2 mg/ml (with or without Triton X-100 [TX-100] to 0.1%), *T. pallidum* was incubated at 33°C for 1 h. Following the addition of phenylmethylsulfonyl fluoride to 1 mg/ml, *T. pallidum* was harvested by centrifugation and probed by immunoblot.

Western blot analysis. Immunoblots were performed as described previously (16) using 1:500 to 1:5,000 dilutions of primary antibody and 1:20,000 to 1:50,000 dilutions of secondary antibody.

Immunofluorescence. *T. pallidum* was encapsulated in agarose beads as described previously (11). Rat α-FlaA and rabbit α-TP0453 sera were diluted 1:100 and added directly to small aliquots of beads and incubated with graded concentrations of Triton X-100 for 2 h with gentle mixing in a 34°C water bath. The beads were pelleted (500 × *g*, 5 min), washed three times with 4 ml of *T. pallidum* cultivation medium, and resuspended in 4 ml of the same. Following the addition of 2 μg of goat α-rat Alexa 546 and 2 μg of goat α-rabbit Alexa 488 conjugates

(Molecular Probes, Eugene, OR), incubation proceeded for an additional 2 h. Washed beads were then viewed on glass slides with a Nikon Optiphot-2 fluorescence microscope equipped with a dark-field condenser and fluorescein and rhodamine filters. For each TX-100 concentration, two slides from each experiment were prepared, and ~100 bacteria/slide were scored for labeling with Alexa 546 and Alexa 488. Differences in labeling at each TX-100 concentration were analyzed by the two-tailed, unpaired, Student's *t* test at the 95% confidence interval. Unencapsulated organisms were stained with the YOYO as described previously (11).

^{14}C-metabolic labeling of lipoproteins. DNA containing 348 bp of upstream sequence and the first 138 bp of coding sequence of TP0453 was PCR amplified with primers TP0453 pho5′ XbaI (5′-GCTCTAGATGGTGCAAAGAATTGA GCGCG-3′) and TP0453 pho3′ BamHI (5′-GCGGATCCCACATCTGCACCA GATCC-3′). The product was digested with XbaI and BamHI, cloned into the same sites of pKS1phoA containing the leader-less alkaline phosphatase gene (*phoA*) of *E. coli* as described previously for GlpQ-PhoA (42), and sequenced. *E. coli* DH5α expressing PhoA fusions with the N terminus of TP0453, LacZ, or GlpQ was metabolically labeled with [^{14}C]palmitic acid as described previously (42). Labeled lysates were probed with α-PhoA monoclonal antibody by immunoblot; the termini of the immunoreactive bands were marked with a pen. Blots were then exposed to film to identify ^{14}C-labeled lipoproteins.

Liposome incorporation assays. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*n*-(cap biotinyl) (DOPE-biotin), 1,2-dimyristoyl-*sn*-glycero-3-phophocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL) in chloroform. Lipids were mixed in defined molar ratios, dried overnight in a laminar flow hood, placed under vacuum for ~2 h, and resuspended to a final concentration of 50 mM lipid.

The ability of rTP0453 to integrate into liposomes was assessed using the pull-down method (45); DOPE-biotin (0.7%, final mol/mol) was added to lipid mixtures consisting of PC or PC:PS:PG (PCSG; 69.3:17:13). Mixtures of 1:1:1 (wt/vol) rTP0453, BR, and rTp47 were added to lipids in 25 mM Tris (pH 7.2), 150 mM NaCl, 2% OG at individual lipid-to-protein ratios of 1,000:1 and dialyzed (Slide-A-Lyzer MINI dialysis unit; Pierce, Rockford, IL) at 4°C overnight against a solution of 20 mM HEPES (pH 7.2), 150 mM NaCl, and BIO-BEADS SM-2 (Bio-Rad). Streptavidin (Sigma) was added to the dialysates to a final streptavidin:biotin ratio of 1:4, and the ionic strength was adjusted to either 0.15 or 1 M with 2 M NaCl in 20 mM HEPES (pH 7.2). Samples were incubated for 30 min at ~23°C followed by centrifugation (20,000 × *g* for 15 min). Supernatants were recovered and centrifuged again to remove liposome carry-over. Pellets were washed once with 10 volumes of buffer of the appropriate ionic strength (0.15 or 1 M NaCl). Both fractions were acetone precipitated, chloroform washed, and dried in a Speed-Vac prior to SDS-PAGE and immunoblot analysis.

Circular dichroism (CD) spectroscopy. To form small unilamellar vesicles (SUVs) for CD spectroscopy of rTP0453 and synthetic peptides, PCSG (70:17:13) lipids were hydrated in CD buffer (5 mM KH_2PO_4 [pH 7.2], 50 mM NaF) for several hours followed by water bath sonication to achieve a clear solution. Far-UV CD spectra were acquired at 25°C on a Jasco J-715 spectropolarimeter (Easton, MD) using a scan rate of 20 nm/min and an 8-s response time. rTP0453, P1A, and synthetic peptides in CD buffer with or without 1% OG were placed in a 1-mm-path-length quartz cuvette and scanned. For measurements taken in the presence of liposomes, freshly prepared SUVs were added to the desired lipid:protein ratio (at least 42:1 for peptides and 1,000:1 to 3,000:1 for proteins) to samples within the quartz cuvette and mixed by inversion immediately prior to CD analysis. Acquired spectra represent the average of 6 to 15 scans from which the background signal (buffer, OG, or SUVs) was subtracted prior to expressing values as mean residue molar ellipticity (MRE, also called θ, measured in degrees per square centimeter per decimole). For proteins, the percentages of helix, sheet, and random coil were calculated from $\theta \times 10^{-3}$ values by K2d (www.embl-heidelburg.de). For peptides, the helical content was calculated using the equation % helix = $\theta_{222}/-39{,}500 + 2.6/n$, where θ_{222} is the MRE value at 222 nm and n is the number of residues (44).

Identification of putative amphipathic α-helices. IMPALA (integral membrane proteins and lipids association) (12) and MacVector (Accelrys, San Diego, CA) were used to analyze residues 26 to 287 of TP0453 for regions with the potential to form amphipathic α-helices. For IMPALA, optimization of peptides was performed by using Hyperchem 5.0 software (geometric optimization using Polak Ribier algorithm with a root mean square gradient of 0.01 kcal/mol Å). A systematic procedure in which all the positions of the center of mass of the peptide along the *z* axis are tested (from −30 Å to 30 Å by steps of 0.5 Å) was applied. For each position of the center of mass, 2,000 angles between the peptide and the interface were tested. The position of minimal energy is retained. Using a window size of nine residues and a periodicity of 100 degrees, the amphipathic α-helix-search function of MacVector was used to screen TP0453 for potential amphipathic α-helices with a hydrophobic moment of 0.6 or greater. Visual inspection of the sequences identified by IMPALA and MacVector on a helical wheel (HelixDraw; www.bioinf.man.ac.uk/~gibson/HelixDraw/helixdraw.html) confirmed that five regions (residues E32 to H42, R130 to D142, N169 to L183, F216 to A228, and S245 to Y257) had potential to form amphipathic α-helices and were subsequently chosen for empirical analysis.

Peptides. N-terminal acetylated and C-terminal amidated peptides based upon the above regions of TP0453 were prepared (95 to 98% purity) by AnaSpec, Inc. (San Jose, CA). Concentrations of freshly prepared solutions in CD buffer were determined either spectrophotometrically as described above or by amino acid analysis performed at the Keck Foundation Amino Acid Analysis and Protein Sequencing Lab at Yale University.

Freeze fracture electron microscopy. PC:PG:DOPE-biotin (68.9:29.5:1.6) liposomes and proteoliposomes (BR and rTP0453 at an initial lipid:protein ratio of 500:1) were prepared by OG dialysis and purified on a 5 to 20% sucrose density gradient as described previously (1). Fractions were dialyzed and assayed for phospholipid and protein content. Dialyzed pure liposome fractions and fractions containing proteoliposomes (550 to 1,000:1 lipid:protein) were pooled separately and cross-linked with streptavidin (8:1 biotin:streptavidin) for 30 min and pelleted. Vesicles were frozen, fractured, and platinum/carbon coated; replicas were examined and photographed with a JOEL 100SX electron microscope as described previously (21).

Membrane fusion assays. Fifty microliters of PCSG liposomes in 20 mM HEPES (pH 7.2), 150 mM NaCl was labeled with 1 μl of the lipophilic fluorescent molecule pKH67 (1 mM in ethanol; Sigma) in the dark for 15 min. rTP0453 in the same buffer was added to a portion of the pKH67-labeled liposomes, resulting in a final lipid concentration of 1 mM and a lipid:protein ratio of 1,000:1. Liposomes were similarly treated with 13.75% polyethylene glycol 8000 as a positive control. Liposome mixtures were incubated for 5 min and examined by fluorescence microscopy at a final magnification of 400×. Matched images for each group were acquired from randomly chosen fields using identical photographic conditions. Buffer or rTP0453-treated SUVs were examined by electron microscopy. rTP0453 in 25 mM Tris (pH 7.3), 25 mM NaCl was quickly added to PCSG SUVs in the same buffer, resulting in final concentrations of 3.81 mM lipid and 3.81 mM rTP0453. Five-milliliter samples of SUVs and TP0453-treated vesicles were absorbed onto formvar-coated, freshly glow-discharged, 200-mesh Cu grids for 1 min and stained for 30 s with 2% uranyl acetate. Samples were examined and photographed on a Philips CM10 transmission electron microscope operating at 60 kV.

Differential scanning calorimetry. To generate (proteo)liposomes, dried lipids (DMPC:DMPG, 70:30) were dissolved in a solution of 10 mM Tris (pH 7.3), 0.15 M NaCl, with our without 3% OG, at a final concentration of 50 mM lipid. rTP0453 (7.5 nmol) (experiments one and two) or 7.5 nmol BR (experiment two) was added to 1.5 mmol lipid in OG and dialyzed against OG-free buffer and BIO-BEADS SM-2 (Bio-Rad) for 3 days. Recovered proteoliposomes were mixed with OG-free liposomes to achieve various lipid-to-protein ratios with a final lipid concentration of 1 mM. In the first experiment, TP0453-containing and free liposomes were incubated at 34°C for 2 h, extruded through a 100-nm pore-size membrane (Avanti Polar Lipids), and degassed prior to analysis; in the second experiment, extrusion was omitted. Measurements were preformed using a VP-DSC microcalorimeter (MicroCal Inc., Northampton, MA) using a thermal gradient of 1.5°C/min from 10°C to 40°C. Transition temperatures and enthalpies were calculated using the Orion software package (MicroCal). In the second experiment, data analysis was trained using BR, which has been shown by differential scanning calorimetry to perturb ~23 lipids per BR molecule (2). Enthalpy data presented here were derived using total, as opposed to membrane-bound, protein.

Calcein release assays. PCSG SUVs were prepared using 50 mM HEPES (pH 7.2) containing 80 mM calcein (Sigma), 100 mM NaCl, 0.3 mM EDTA to a final lipid concentration of 50 mM. Calcein-loaded SUVs were separated from free calcein using a P6 column (Bio-Rad) with 25 mM Tris (pH 7.3), 150 mM NaCl as the mobile phase and used within 2 h. Proteins (rTP0453, TP0453-MBPH6, and MBPH6) or polyethylene glycol in the same buffer was added to the wells of 96-well plates (Costar 3925; Corning Inc., Corning, N.Y.), after which calcein-loaded SUVs were added to a final lipid concentration of 10 mM. After mixing and 10 min of incubation, fluorescence emission was measured at 530 nm with a PerSeptive Biosystems CytoFlour 4000 plate-reading spectrofluorometer using an excitation wavelength of 485 nm. Measurements in which buffer or 1% OG were used in place of protein were used to determine the values for baseline and

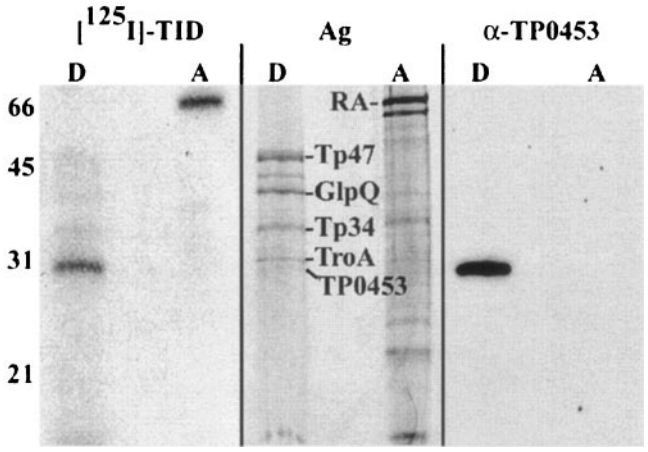

FIG. 1. TP0453 is an unabundant, amphiphilic OMP. [^{125}I]TID-labeled *T. pallidum* cells were Triton X-114 phase partitioned; the detergent-enriched (D) and aqueous (A) phases were resolved by SDS-PAGE for autoradiography ([^{125}I]TID), silver staining (Ag), or immunoblot analysis (α-TP0453). As reported for ovalbumin (19), rabbit albumin (RA) also was [^{125}I]TID labeled.

complete calcein release, respectively. Fluorescence values were baseline subtracted and expressed as a percentage of maximum calcein release.

RESULTS

TP0453 is a *T. pallidum* OMP. While well-established methods exist for the identification of OMPs of gram-negative bacteria, efforts to apply these techniques to *T. pallidum* have proven unsuccessful due to the inability to cultivate the bacterium in vitro and the fragility of its OM (37, 42). As an alternative strategy, we utilized the highly apolar probe 3-(trifluoromethyl-)-3-(*m*-[^{125}I]iodophenyl-diazarene) ([^{125}I]TID), which stably partitions into the hydrocarbon core of a membrane and, upon UV photoactivation, promiscuously binds to any intramembranous constituents (6, 42). In the case of an intact, double-membrane organism, such as *T. pallidum*, [^{125}I]TID should become trapped in the OM. As this probe will label any membrane protein that it gains access to (6), great care was taken to avoid compromising the integrity of the OM. Triton X-114 phase partitioning (5), which segregates hydrophilic and amphiphilic (membrane) proteins into aqueous and detergent phases, respectively, was applied to [^{125}I]TID-labeled *T. pallidum*. A single, strongly radiolabeled polypeptide of ~30.5 kDa was detected within the detergent-enriched phase (Fig. 1). The labeling was OM specific, as relatively abundant inner membrane (IM) proteins, such as Tp47, GlpQ, and TroA (1, 42), were unlabeled (Fig. 1). By resolving detergent-phase extracts on a two-dimensional gel and sequencing internal peptides, the labeled spot was identified as the product of *T. pallidum* gene number 453 (TP0453), which computer algorithms predict to be an OMP (7). Consistent with an OM location, the sequence of the putative mature (i.e., processed) protein lacks long stretches of hydrophobic amino acids that would serve as IM-spanning domains (22). With the exception of an ortholog (TDE2662; 32%/55% amino acid identity/similarity) in the genome of the related oral spirochete *T. denticola* (41), TP0453 has no homology with any protein in the databases.

TP0453 lacks surface-exposed domains. Experiments were next performed to detect extracellular domains of TP0453. Incubation of *T. pallidum* with proteinase K did not discernibly affect either TP0453 or the periplasmic flagellar protein, FlaA, unless the treponemes were treated with 0.1% Triton X-100 (Fig. 2A), a concentration that solubilizes the OM (11). To more precisely localize TP0453, we used a mixture of α-TP0453 and α-FlaA polyclonal antibodies to perform double-label immunofluorescence microscopy of *T. pallidum* encapsulated in porous agarose beads. Encapsulation minimizes damage to the bacterium's fragile OM during labeling studies (11) and also allows for the localization of subsurface proteins following the addition of detergent; inclusion of α-FlaA antiserum allows for the identification of bacteria with grossly damaged OMs. In the absence of Triton X-100, an average of 92% of treponemes were unlabeled by either antisera (Fig. 2B and C); of the labeled fraction, approximately 6% were double labeled, whereas 2% were labeled by α-TP0453 antiserum alone. Subcritical micellar concentrations of Triton X-100 (i.e., <0.02%), which would be expected to preferentially perturb the outer leaflet of the OM (17), markedly increased the percentage of treponemes labeled by α-TP0453 antibodies without a corresponding increase in labeling of FlaA (Fig. 2B and C). In parallel experiments with unencapsulated *T. pallidum*, similarly detergent-treated spirochetes remained largely motile and excluded the fluorescent dye YOYO (molecular weight, 1,270) (data not shown), further indicating that the OMs were not grossly compromised. At Triton X-100 concentrations greater than 0.02%, FlaA and YOYO labeling became appreciable and *T. pallidum* became nonmotile. Nevertheless, labeling of TP0453 was significantly greater than that of FlaA until organisms were exposed to 0.08% Triton X-100 (Fig. 2B and C). Taken as a whole, the data presented thus far indicate that TP0453 is an OMP with intramembranous but not surface-exposed domains. Based upon the above observations and the topological data presented below, we believe that the small percentage of spirochetes selectively labeled by α-TP0453 antiserum in the absence of detergent likely represent organisms whose OMs suffered minor perturbations during encapsulation.

TP0453 is a lipid-modified, integral membrane protein. Although not annotated as a lipoprotein in the genomic sequence (13), TP0453 contains a putative signal peptide terminated by a potential lipobox (LFASC). By metabolically labeling an N-terminal TP0453-alkaline phosphatase fusion protein with [^{14}C]palmitate in *E. coli*, we confirmed that the TP0453 signal peptide directs export and lipid modification (Fig. 3A). All spirochetal lipoproteins studied thus far consist of a membrane-anchoring lipid moiety that confers amphiphilicity to a hydrophilic protein (21). In contrast to a nonlipidated form of a well-characterized *T. pallidum* lipoprotein (Tp47) (47), recombinant, nonlipidated TP0453 (rTP0453) was predominantly amphiphilic as determined by Triton X-114 phase partitioning (Fig. 3B). Interestingly, rTP0453 was not as amphiphilic as its native (lipidated) counterpart (Fig. 1) or the polytopic TM protein bacteriorhodopsin (BR) (Fig. 3B), both of which partition exclusively into the detergent-enriched phase. Proteins possessing the capacity to exist in both soluble

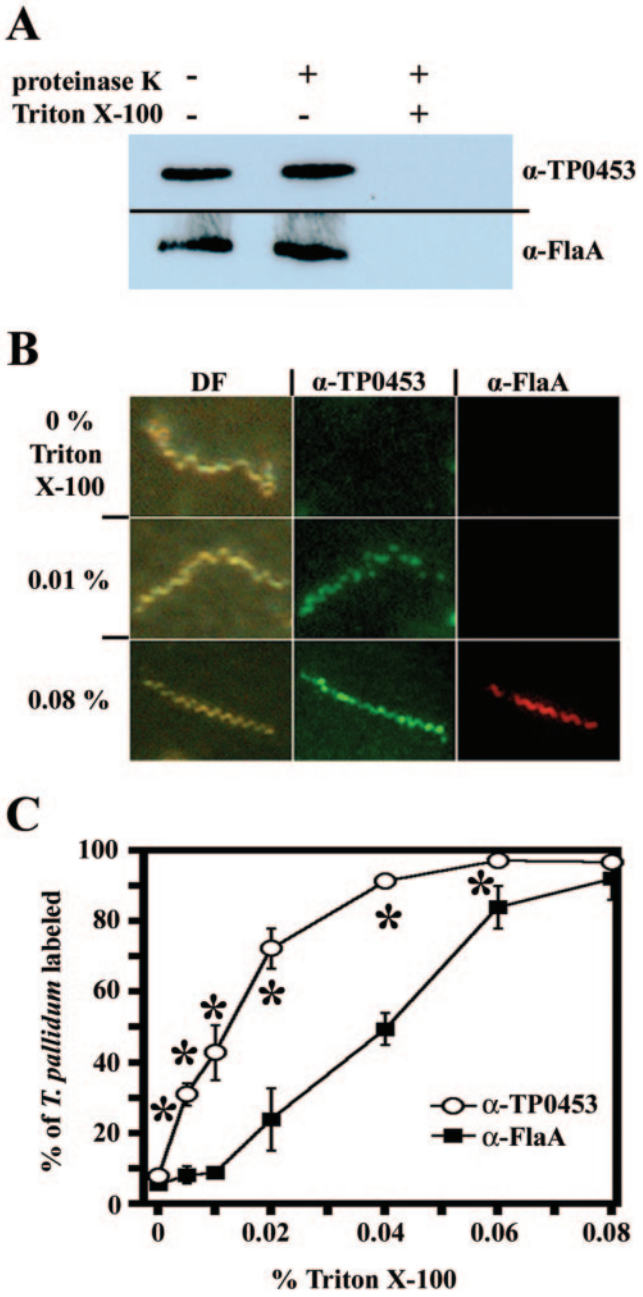

FIG. 2. TP0453 lacks surface exposure. (A) *T. pallidum* was exposed to PBS, proteinase K, or 0.1% Triton X-100 plus proteinase K and subsequently analyzed for TP0453 and FlaA by immunoblot. (B and C) *T. pallidum* encapsulated in agarose beads was probed simultaneously with rabbit α-TP0453 and rat α-FlaA antisera in the presence of increasing concentrations of Triton X-100. Following exposure to Alexa 488 (green) and Alexa 546 (red) α-rabbit and α-rat immunoglobulin conjugates, individual *T. pallidum* cells were examined by dark-field (DF) and fluorescence microscopy. The percentage of labeled spirochetes at each Triton X-100 concentration is plotted; asterisks indicate differences considered significant ($P < 0.01$) by the Student's unpaired, two-tailed *t* test; labeling of each antigen at each detergent concentration was independently determined a minimum of three times. Error bars are standard deviations.

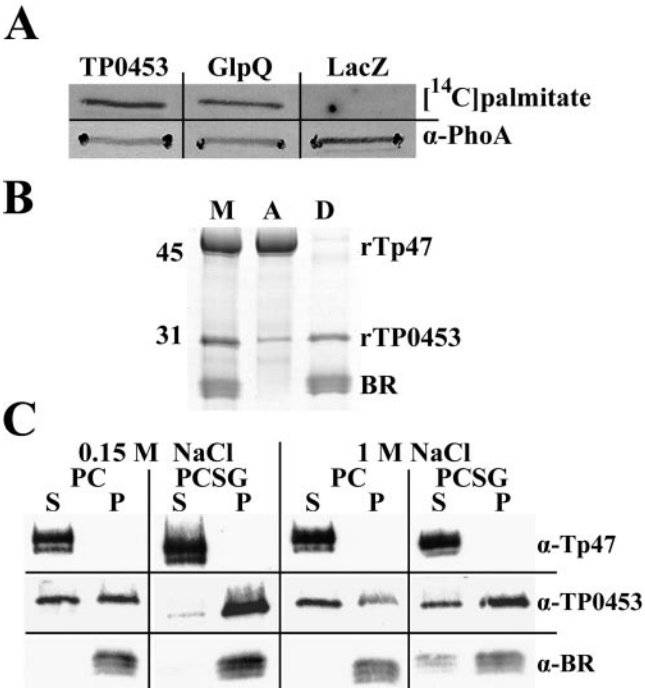

FIG. 3. TP0453 is a lipid-modified, integral membrane protein. (A) *E. coli* cells expressing fusions between a leaderless PhoA with the N termini of TP0453, the *T. pallidum* lipoprotein GlpQ (42), or LacZ were metabolically labeled with [^{14}C]palmitate and analyzed by immunoblotting using an α-PhoA monoclonal antibody prior to autoradiography. The termini of the immunoreactive bands were marked with a pen. (B) A mixture (M) of rTp47, rTP0453, and BR was fractionated into Triton X-114 aqueous (A) and detergent (D) phases, resolved by SDS-PAGE, and stained with Coomassie blue. (C) Liposomes consisting of PC or PCSG were generated in the presence of a mixture of rTp47, rTP0453, and BR. After adjusting the ionic strength to either 0.15 M or 1 M NaCl, the proteoliposomes were harvested and the resulting pellets (P) and supernatants (S) were probed by immunoblotting with the indicated antisera.

and membrane-integrated forms have been termed amphitropic (20). To further characterize the amphitropic properties of TP0453, we examined the ability of rTP0453 to associate with liposomes (45). Consistent with the above findings, the amphiphilicity of rTP0453 observed in the presence of zwitterionic, phosphatidylcholine (PC) liposomes under physiologically relevant salt conditions (0.15 M NaCl) was greater than that of rTp47 and less than that of BR (Fig. 3C). Binding of rTP0453 to anionic liposomes (69.3% PC, 17% phosphatidylserine, and 13% phosphatidylglycerol [PCSG], which simulate the phospholipid composition of the *T. pallidum* OM [39]) was markedly enhanced over that observed with PC liposomes (Fig. 3C). Significantly, the association of rTP0453 with anionic membranes was largely resistant to a high-salt (1 M NaCl) wash (Fig. 3C), a hallmark of integral membrane proteins.

TP0453 is a monomer lacking extensive β-sheet structure. In contrast to the hydrophobic α-helical TM segments of IM proteins, all bacterial integral OMPs of known structure span the membrane via a series of antiparallel β-strands arranged to form a β-barrel (22). However, the lack of surface exposure of native TP0453 and the amphitropic nature of the recombinant protein seemed to be inconsistent with this topology. There-

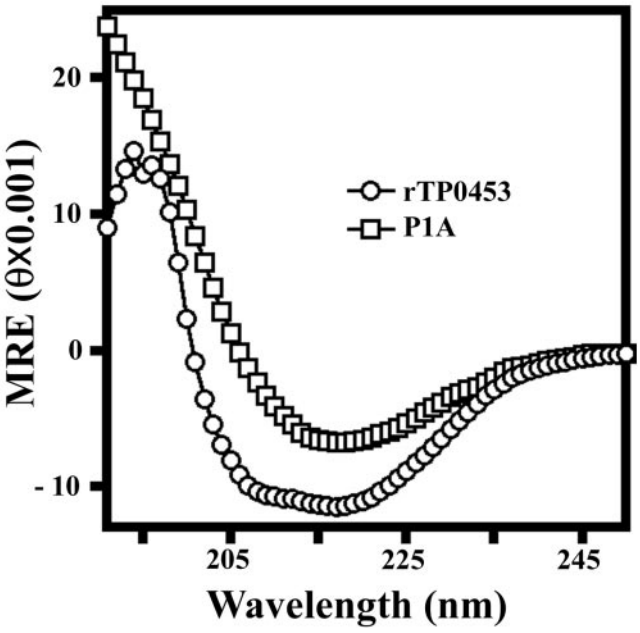

FIG. 4. CD spectroscopy of rTP0453 and P1A indicates that rTP0453 lacks extensive β-sheet structure. A single minimum near 217 nm indicates β-sheet structure, whereas double minima around 208 and 220 nm coupled with a maximum around 193 nm indicate α-helical structure.

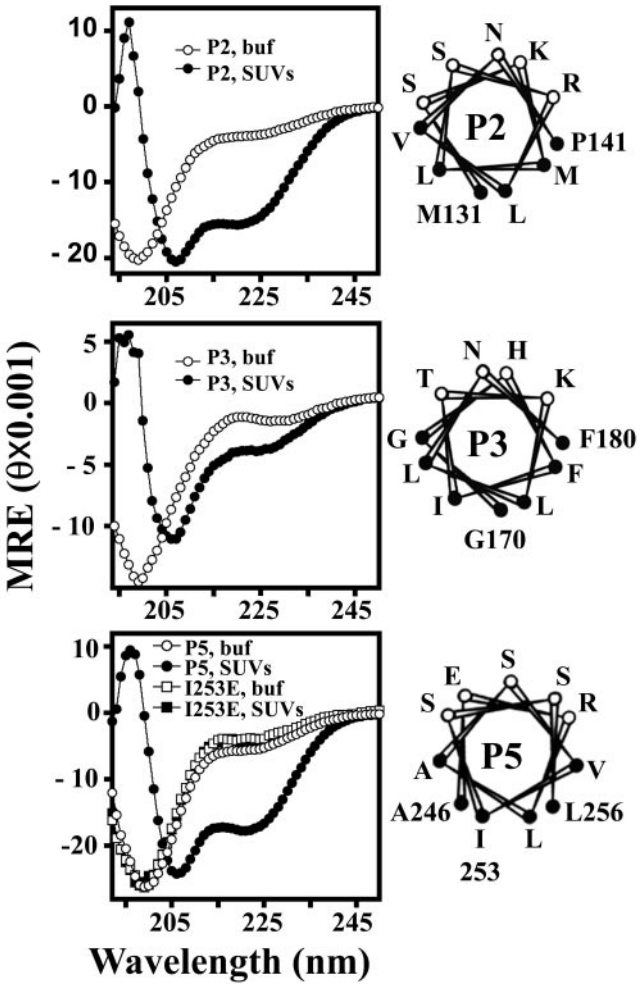

FIG. 5. TP0453 contains multiple amphipathic α-helices. CD spectra and helical wheel projections of peptides P2 (130 to 142), P3 (169 to 183), P5 (245 to 257) (all circles), and I253E (squares) in the absence (open symbols, buffer [buf]) and presence (filled symbols) of PCSG small unilamellar vesicles (SUVs). A minimum around 199 nm indicates a lack of CD-detectable structure. Note that the spectra of P5 and I253E in buffer and I253E in SUVs are superimposable. In the helical wheel projections, polar and hydrophilic residues are indicated with open symbols and nonpolar and hydrophobic residues are shown as filled symbols; note that not all residues are shown.

fore, circular dichroism (CD) spectroscopy was used to determine if TP0453 has extensive β-sheet structure. In marked contrast to the gonococcal porin P1A, which has a characteristic β-sheet CD spectrum (average of 59% β-sheet and 13% α-helix in the presence of 1% octylglucoside [OG]), the spectrum of rTP0453 indicated a lesser degree of β-sheet and a pronounced α-helical content (average of 18% β-sheet and 28% α-helix) (Fig. 4). Attempts to perform these measurements in the presence of lipid bilayers were hindered by the propensity of rTP0453 to induce fusion of liposomes (data not shown). As many OMPs form multimers, the oligomeric state of rTP0453 was examined by size-exclusion chromatography. In 1% OG, rTP0453 eluted as a single peak with a retention time identical to that of the globular ovalbumin (44 kDa) (data not shown), a finding most consistent with a monomer (29.1 kDa) in an extended, nonglobular conformation bound to an OG micelle (8 kDa) (18).

TP0453 contains membrane-inserting amphipathic α-helices that do not traverse the bilayer. As TP0453 lacks both long stretches of hydrophobic amino acids found in IM proteins and the extensive β-sheet structure found in all integral OMPs of known structure, we considered that it might be integrating into the OM via one or more amphipathic α-helices, a structural motif in which hydrophilic and hydrophobic residues are found on opposite sides of an α-helix (20). While such architecture lacks precedent among OMPs, it is consistent with the amphitropic nature of rTP0453 and the protein's lack of surface exposure. Two computer-based programs, IMPALA (12) and MacVector, were used to search the sequence of the mature protein for amphipathic α-helices that then were examined further using a helical wheel prediction. This search identified five potential amphipathic α-helices corresponding to residues 32 to 42, 130 to 142, 169 to 183, 216 to 228, and 245 to 257 (P1 through P5, respectively), which are predicted to lie relatively flat (1.5 to 13.5 degrees off parallel) within one leaflet of the membrane, displacing phospholipid head groups and entering the apolar hydrocarbon core (data not shown). As peptides derived from amphipathic α-helices are frequently unstructured in aqueous environments and helical in the presence of membranes (44), we used CD to examine the secondary structures of peptides corresponding to the putative amphipathic α-helix regions of TP0453. P1 and P4 presented solubility problems that precluded empirical analysis. P2, P3, and P5 were unstructured in aqueous buffer yet adopted various degrees of helical structure (60, 27, and 65%, respectively) upon the addition of anionic small unilamellar vesicles (SUVs)

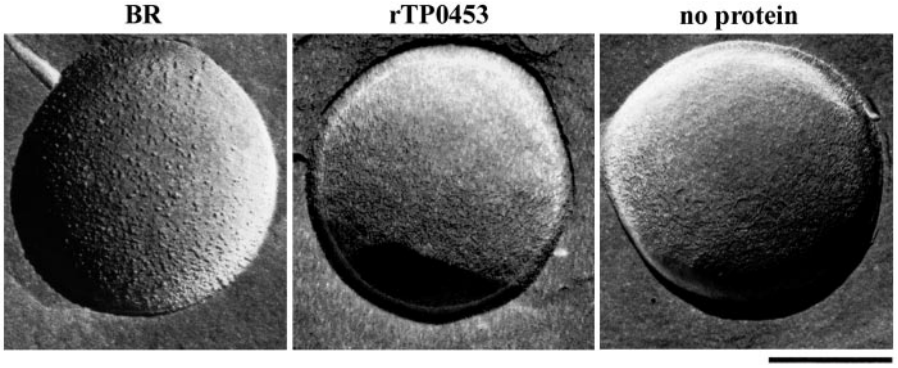

FIG. 6. rTP0453 is not a TM protein. Sucrose gradient-purified liposomes containing BR (lipid to protein, ≥1,000:1), rTP0453 (lipid to protein, ≥550:1), or no protein visualized by freeze fracture electron microscopy. TM proteins, such as BR, form intramembranous particles that can be visualized by freeze fracture electron microscopy, whereas membrane proteins that occupy only a single leaflet of the bilayer do not (43). Bar, 0.2 μm.

(Fig. 5). A variant of P5, I253E, in which an isoleucine predicted to reside on the hydrophobic face of the helix was replaced by the charged residue glutamic acid, remained unstructured in the presence of SUVs (Fig. 5).

TM proteins form intramembranous particles that can be visualized by freeze fracture electron microscopy, whereas membrane proteins that occupy only a single leaflet of the bilayer do not (43). We therefore used this technique to further examine the membrane topology of rTP0453. In striking contrast to BR proteoliposomes, which were studded with particles, rTP0453-loaded vesicles were slightly rougher than protein-free liposomes but lacked discernible particles (Fig. 6).

TP0453 decreases membrane integrity and facilitates TM flux of hydrophilic solutes. Amphipathic α-helix motifs in a number of peptides and proteins have been shown to destabilize lipid bilayers (31, 43); as mentioned earlier in our CD studies, rTP0453 induces liposome fusion (data not shown), a process which requires membrane destabilization. To examine more quantitatively the capacity of this protein to affect membrane structure, differential scanning calorimetry was used to measure the influence of rTP0453 on the organization of lamellar phospholipids; decreases in transition enthalpy indicate decreased lipid order. Both BR and, to a much greater extent, rTP0453, induced concentration-dependent decreases in the transition enthalpy of anionic membranes (Fig. 7A, inset), further demonstrating that TP0453 is an integral membrane protein (2). Additionally, rTP0453 induced a slight reduction of the lipid transition temperature (Fig. 7A, inset). In plots of transition enthalpy versus protein-to-lipid ratio, the *x*-axis intercept indicates the number of lipids perturbed by each protein molecule (2, 43). The values for BR and rTP0453 are 28 and an average of 184, respectively, (Fig. 7A); of note, BR was previously shown by differential scanning calorimetry to perturb ~23 lipids (2). Notably, a value this high for a protein the size of rTP0453 (29.1 kDa) is inconsistent with a globular, TM topology and instead points to an extended conformation within the plane of the membrane (43). To determine whether membrane perturbation by rTP0453 could promote TM flux of hydrophilic solutes, we assayed for release of the small (623 Da) fluorescent marker, calcein, encapsulated within liposomes. As shown in Fig. 7B, rTP0453 promoted calcein release in a dose-dependent manner with activity detected at lipid-to-protein ratios as high as 500:1. Highly similar results were obtained with a TP0453 fusion protein (rTP0453-MBPH6) that was purified in the absence of detergents. Neither the MBPH6 fusion partner nor the fusogenic agent polyethylene glycol induced discernible calcein release (Fig. 7B).

DISCUSSION

Ten years ago, in an effort to facilitate the identification of syphilis vaccine candidates, we enumerated properties which a bona fide *T. pallidum* rare OMP ought to posses (37). TP0453 has three of these: it is unabundant and amphiphilic, and it lacks long stretches of hydrophobic residues in the mature form. However, we also assumed that a *T. pallidum* OMP would be a nonlipidated, TM protein with surface-exposed domains. Unexpectedly, all of our data are most consistent with a topology in which the peptide moiety of the TP0453 lipoprotein inserts into the periplasmic leaflet of the OM but does not traverse the membrane to become surface exposed (Fig. 8). As all integral OMPs of known structure are surface-exposed, TM β-barrel proteins (22), TP0453 appears to be a novel type of OMP.

The absence of orthologs for gram-negative porins and OM nutrient-specific transporters (13) in the *T. pallidum* genome predicts that transport of nutrients across the OM must differ fundamentally in the very slow-growing *T. pallidum* and more rapidly dividing gram-negative bacteria. Although the genetic intractability of *T. pallidum* precludes analyzing the contribution of TP0453 to nutrient uptake in vivo, based upon the capacity of the recombinant protein to perturb membranes and promote TM solute flux we hypothesize that TP0453 may render the *T. pallidum* OM permeable to a variety of nutrients. It should be noted that previous reports of a *T. pallidum* protein (TroA) having porin activity (4) have been discounted (1, 15, 25, 26). As such, the means by which nutrients traverse the *T. pallidum* OM have remained enigmatic. While the mechanism(s) by which TP0453 increases membrane permeability remains to be determined, we hypothesize that the amphipathic α-helices induce localized, transient bilayer discontinuities sufficient to allow nonselective transit of small hydrophilic

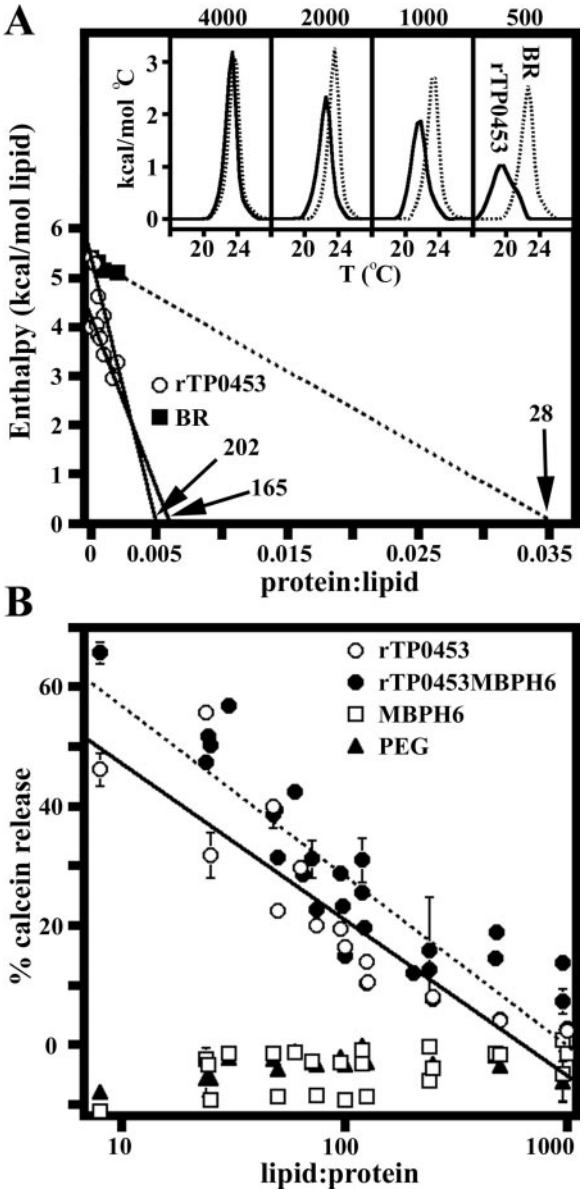

FIG. 7. rTP0453 perturbs membranes to facilitate solute flux. (A) Differential scanning calorimetry analysis of liposomes and BR and rTP0453 proteoliposomes; two independent determinations were performed for rTP0453. The inset shows thermograms of rTP0453 (solid lines and open circles) and BR (dashed lines and filled squares) proteoliposomes at the lipid-to-protein ratios indicated above each thermogram. Enthalpies in the main figure were derived from the areas under the thermographic curves; arrows indicate the lipid-to-protein ratios at which the enthalpy becomes zero. (B) Release of calcein from SUVs exposed to TP0453. SUVs loaded with calcein at a self-quenching concentration were treated with proteins (rTP0453, open circles; rTP0453MBPH6, filled circles; MBPH6, open squares) at the lipid-to-protein ratios indicated on the *x* axis or polyethylene glycol (PEG, filled triangles) at final concentrations of 13.75%, 6.88%, 3.44%, 1.72%, 0.86%, 0.43%, 0.22%, or 0.11%. Fluorescence resulting from released (dequenched) calcein was quantified and normalized against complete release induced by OG. Results are from four independent experiments; lines of best fit are indicated for rTP0453 (solid) and rTP0453MBPH6 (dashed). Error bars are standard deviations.

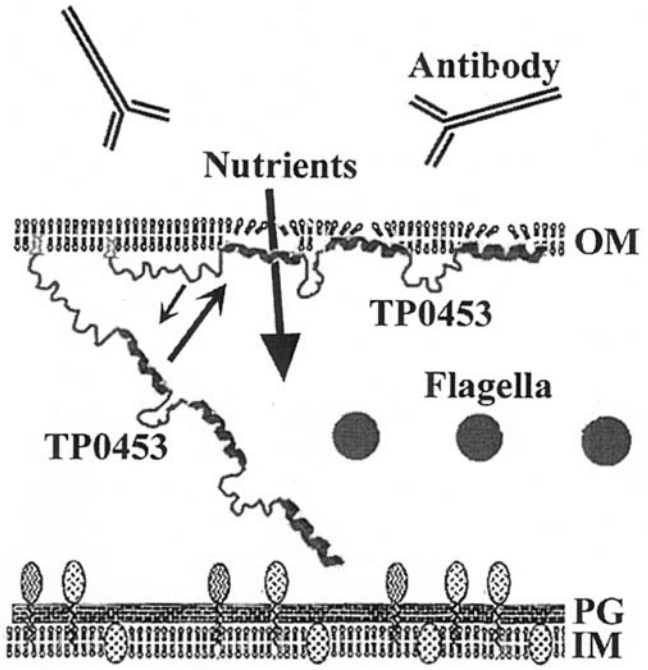

FIG. 8. A model depicting the proposed topology and membrane perturbation activity of TP0453 in the *T. pallidum* OM. We hypothesize that the multiple amphipathic α-helices (three shown) present in TP0453 allow the amphitropic peptide moiety of the lipid-tethered protein to shuttle between the periplasm and the periplasmic leaflet of the OM. Based upon the membrane-perturbing capacity of rTP0453 (see Fig. 7), we propose that the native protein functions to facilitate nonspecific solute (nutrient) flux without becoming accessible to specific antibodies.

molecules (e.g., nutrients) (Fig. 8). We propose that the low abundance of native TP0453, in concert with a dynamic equilibrium between the OM-inserted and periplasmic forms of the lipoprotein's peptide moiety, serves to limit the degree of OM perturbation and maintain the spirochete's low surface antigenicity.

Although the concealed topology of TP0453 was unexpected, the resulting antibody inaccessibility is very consistent with the parasitic strategy of *T. pallidum*, which relies upon resistance to specific antibody binding as a primary mechanism for immune evasion. In gram-negative bacteria the surface-exposed portions of OMPs are targets of phages, colicins, and antibodies (22, 35). To minimize the impact of these agents, many extracellular bacteria have developed active counter measures, such as antiphagocytic capsular polysaccharides, antigenic variation of OMPs, expression of proteases that cleave immune components, etc. Evolution appears to have taken a different tack with *T. pallidum*, which only minimally, if at all, invokes the benefits and liabilities associated with surface-exposed proteins. Notably, α-TP0453 antibodies, which would lack access to the antigen, have been shown to be present in all stages of *T. pallidum* infection (46). It is conceivable that the bacterium's exceptionally slow growth rate is a consequence of an "evolutionary trade-off" in which antibody-accessible porins and OM nutrient-specific transporters associated with rapid growth were eliminated in favor of reducing the bacterium's susceptibility to antibody.

Intramembranous particles observed in the *T. pallidum* OM by freeze fracture electron microscopy have long been presumed to represent one or more rare TM proteins (38, 46a). Our findings with liposomes indicate that rTP0453 is not a particle-forming protein. We recognize that the recombinant protein lacks the lipid moiety found in the native lipoprotein. However, as lipid modification has little effect on the topology of spirochetal lipoproteins (21, 42, 47), it is thermodynamically unlikely that the addition of a lipid moiety could change TP0453 from an amphipathic α-helix-containing protein in an extended, nonglobular configuration to the transmembrane β-barrel structure found in all integral OMPs of known structure. Analysis of the native ortholog in *T. denticola* (TDE2662), a predicted lipoprotein with several putative amphipathic α-helices, will enable this topology to be tested in a native context. If our conception of TP0453 is correct, the particles in the *T. pallidum* OM most likely represent a distinct OMP of such exceedingly low abundance as to be below the level of detection of [^{125}I]TID labeling, or perhaps less likely, a nonproteinaceous structure. One protein, TprK, has received much attention as a putative OMP (8–10, 14, 23, 24, 32–34), although its OM location has been challenged (16). Three other proteins, TprF, TprI, and Tp92, appear to be viable candidates that merit rigorous empirical analysis (7). Speculation that the *T. pallidum* OM particles may represent nonproteinaceous entities is fostered by anomalies in the freeze fracture electron microscopy behavior of these entities. TM proteins examined by freeze fracture electron microscopy typically show (i) temperature-induced aggregation in response to lipid phase separations, (ii) proportionality in the numbers of particles and pits, and (iii) in matched faces, particle-to-pit complementarity (27). In contrast to the particles present in the protein-rich, *T. pallidum* IM which exhibit these typical behaviors, the OM particles do not (38). Identification and characterization of these particles, proteinaceous or not, remains a formidable challenge.

As many previously characterized *T. pallidum* lipoproteins have been definitively localized to the IM, the observation that TP0453 is an OM lipoprotein suggests the existence of a functional lipoprotein sorting system in *T. pallidum*. In gram-negative bacteria, lipoproteins containing a D residue immediately after the acetylated C residue (the "Lol avoidance" signal) are retained in the IM, and all others are passed from the IM-bound LolCDE complex to the soluble periplasmic chaperone LolA to the OM-bound receptor LolB and finally to the OM (30). While orthologs of LolC, LolD, LolE, and potentially LolA are present in the *T. pallidum* genome, an ortholog of LolB, which inserts the lipoprotein into the periplasmic leaflet of the OM, is lacking (13). Moreover, the Lol avoidance signal is absent from both TP0453 and the well-characterized *T. pallidum* IM lipoproteins Tp47, GlpQ, Tp15, and Tp17. Thus, it is likely that the Lol system in *T. pallidum* functions somewhat differently than in gram-negative bacteria. Interestingly, TP0453 does have a 4-residue motif, GAWK, starting at position +4 (the acetylated C residue is +1), which is lacking in the IM lipoproteins. Perhaps this motif functions as a *T. pallidum* "Lol acceptance" signal allowing for engagement by the LolCDE complex. The amphiphilicity of the TP0453 protein moiety may enable LolA to release this lipoprotein directly into the OM, thereby obviating the need for LolB.

In this report, we describe an OMP that contains multiple amphipathic α-helices that insert into the periplasmic leaflet of the OM which shields the protein from antibody. As such, the architecture of TP0453 departs radically from that of all integral OMPs of known structure which span the OM via amphipathic β-barrels (22). The capacity of rTP0453 to perturb a large number of OM lipids and induce flux of small hydrophilic molecules in vitro prompts us to hypothesize that the protein's function in vivo may be to promote transient, localized bilayer discontinuities which allow nonselective nutrient uptake. While novel, this model of TP0453 promoting solute transit across the OM without being antibody accessible is strikingly consistent with the parasitic strategy of this very slow-growing, obligate pathogen.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI-10573 (K.R.O.H.) and AI-26756 (J.D.R.). M.D. is supported by the Belgian program on Interuniversity Pole of Attraction (PAI #P5/33), and R.B. is an FNRS Research Director.

We are grateful to Dmitriy Shevchenko for technical assistance, to G. King, A. Das, P. Yeagle, D. Akins, E. Fikrig, M. Caimano, and C. Eggers for critical evaluation of the manuscript, and to A. Albert for providing access to DSC instrumentation.

The authors have no conflicting financial interests.

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