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Tromp1, a Putative Rare Outer Membrane Protein, Is Anchored by an Uncleaved Signal Sequence to the *Treponema pallidum* Cytoplasmic Membrane

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Treponema pallidum **rare outer membrane protein 1 (Tromp1) has extensive sequence homology with substrate-binding proteins of ATP-binding cassette transporters. Because such proteins typically are periplasmic or cytoplasmic membrane associated, experiments were conducted to clarify Tromp1's physicochemical properties and cellular location in *T. pallidum*. Comparison of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis mobilities of (i) native Tromp1 and Tromp1 synthesized by coupled in vitro transcription-translation and (ii) native Tromp1 and recombinant Tromp1 lacking the N-terminal signal sequence revealed that the native protein is not processed. Other studies demonstrated that recombinant Tromp1 lacks three basic porin-like properties: (i) the ability to form aqueous channels in liposomes which permit the influx of small hydrophilic solutes, (ii) an extensive β-sheet secondary structure, and (iii) amphiphilicity. Subsurface localization of native Tromp1 was demonstrated by immunofluorescence analysis of treponemes encapsulated in gel microdroplets, while opsonization assays failed to detect surface-exposed Tromp1. Incubation of motile treponemes with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazarine, a photoactivatable, lipophilic probe, also did not result in the detection of Tromp1 within the outer membranes of intact treponemes but, instead, resulted in the labeling of a basic 30.5-kDa presumptive outer membrane protein. Finally, analysis of fractionated treponemes revealed that native Tromp1 is associated predominantly with cell cylinders. These findings comprise a body of evidence that Tromp1 actually is anchored by an uncleaved signal sequence to the periplasmic face of the *T. pallidum* cytoplasmic membrane, where it likely subserves a transport-related function.**

Venereal syphilis is a chronic, multisystem infectious disorder caused by the spirochetal bacterium *Treponema pallidum* subsp. *pallidum* (*T. pallidum*). Like all spirochetes, *T. pallidum* is an elongated, highly motile organism that consists of a fragile outer membrane surrounding a periplasmic space, a peptidoglycan-cytoplasmic membrane (PG-CM) complex, and a protoplasmic cylinder (36). There is now a substantial body of evidence that the outer membranes of *T. pallidum* and enteric gram-negative bacteria differ considerably with respect to composition and molecular architecture (52, 57). One of the key differences concerns the relative abundance of proteins with membrane-spanning domains. Whereas outer membranes of gram-negative bacteria contain high densities of such polypeptides (48), freeze-fracture electron microscopy and cell fractionation studies have shown that they are sparse in *T. pallidum*, hence the designation rare outer membrane proteins (52, 57, 59, 77).

Based on the assumption that *T. pallidum* rare outer membrane proteins are important in disease pathogenesis, molecular characterization of these polypeptides has become a major objective of contemporary syphilis research. One strategy recently developed to accomplish this goal is to isolate *T. pallidum* outer membranes for partial amino acid sequencing of candidate rare outer membrane proteins (9, 60). Using this approach, Blanco et al. (6) identified a 31-kDa protein (Tromp1) in isolated outer membranes which formed ion-conducting channels in planar lipid bilayers. More recently, the same investigators reported that recombinant Tromp1 expressed in *Escherichia coli* is surface exposed and that the recombinant protein possesses porin-like properties (7). However, to date, no evidence for either outer membrane location or surface exposure of native Tromp1 within motile or intact treponemes has been presented (6, 7). Moreover, Tromp1 has extensive sequence homology with the periplasmic substrate-binding proteins of known ATP-binding cassette (ABC) transporters and the *tromp1* gene is transcriptionally linked to open reading frames which encode homologs for ABC transporter components which, in gram-negative bacteria, are cytoplasmic membrane associated (30, 33). The apparent discrepancy between the studies of Blanco and coworkers (6, 7) and these newer genetic data prompted efforts to clarify the physicochemical properties and cellular location of Tromp1 in *T. pallidum*. Here we present evidence that Tromp1, rather than being an outer membrane-spanning protein, actually is anchored by an uncleaved signal sequence to the *T. pallidum* cytoplasmic membrane.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *T. pallidum* subsp. *pallidum* (Nichols strain) was passaged by intratesticular inoculation of New Zealand White rabbits as previously described (58). *E. coli* DH5α (Gibco/BRL, Gaithersburg, Md.) was used for transformation experiments. Except where otherwise stated, strains and

transformants were grown on yeast-tryptone agar or broth supplemented with the appropriate antibiotic. Plasmid pGEX-4T-2 (68) (Pharmacia LKB Biotechnology, Piscataway, N.J.) was used for generating a Tromp1–glutathione *S*-transferase (GST) fusion protein. The pCRII vector (Invitrogen, San Diego, Calif.) was used for cloning PCR products. pSKI/pho (54) was used to construct a Tromp1-alkaline phosphatase (PhoA) fusion for processing experiments.

Pulse-chase processing. Processing experiments were performed with *E. coli* maxicells as previously described (19).

In vitro-coupled transcription-translation. DNA fragments containing the complete *tromp1* and *flaA* genes and promoter regions were amplified by PCR with *T. pallidum* DNA as the template and the following primer pairs: forward primer 5′-TGCTTATGCGAGGAAGATCAAA-3′ and reverse primer 5′-CCC ACTCAACTCTACATTCCA-3′, which were used to amplify *tromp1*, and forward primer 5′-TTTGAAAGTGATGGGTAGAT-3′ and reverse primer 5′-CTACTGCTGCTCTTCCTGCCG-3′, which were used to amplify *flaA*. Four micrograms of each PCR product was gel purified and used for in vitro transcription-translation with the *E. coli* S30 extract for linear templates (Promega, Madison, Wis.) and [^{35}S]methionine (Amersham, Arlington Heights, Ill.) according to Promega's instructions. Five microliters of each reaction mixture was acetone precipitated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose for immunoblotting with rat anti-Tromp1 antiserum or monoclonal antibody (MAb) H9-2. *T. pallidum* whole-cell lysates were run in a lane adjacent to the radiolabeled material to compare the relative SDS-PAGE mobilities of the native Tromp1 and FlaA polypeptides (visualized by immunoblotting) with the in vitro-radiolabeled products. After immunoblot analysis, the nitrocellulose filters were exposed to film, which was needle punched in several places to precisely orient the film with respect to the immunoblot.

Production of Tromp1 fusion proteins. A cleavable Tromp1-GST fusion was constructed by cloning a DNA fragment encoding amino acids 41 to 318 of the reported Tromp1 sequence (6) into the *Bam*HI and *Eco*RI sites of the pGEX-4T-2 polylinker. The DNA fragment was generated by PCR with *T. pallidum* DNA as the template and the following primers: 5′-CGGGATCCGACGGGA AACCCCTGGTTGTC-3′ (*Bam*HI site plus nucleotides 247 to 267) and 5′-CGGAATTCCTAGCGAGCCAACGCAGCAACG-3′ (*Eco*RI site plus nucleotides complementary to bases 1083 to 1062). The resulting Tromp1-GST fusion was purified by affinity chromatography on an agarose-glutathione matrix according to the manufacturer's (Pharmacia LKB Biotechnology) instructions. The Tromp1 portion was cleaved from the fusion with thrombin as previously described (78).

A Tromp1-alkaline phosphatase (PhoA) fusion was constructed in the vector pSKI/pho (54) as follows. A PCR product was generated with *T. pallidum* DNA and primers 5′-GCTCTAGATGCTTATGCGAGGAAGATCAAATG-3′ (nucleotides 1 to 24 plus *Xba*I site) and 5′-CGGGATCCGCCAATGGTGGTGAC AACCAGGGG-3′ (complementary to nucleotides 279 to 256 plus *Bam*HI site). The resulting product was digested to completion with the restriction enzymes *Xba*I and *Bam*HI and cloned into the pSKI/pho polylinker.

Immunological reagents. To generate polyclonal antisera directed against Tromp1, Sprague-Dawley rats and New Zealand White rabbits were primed by intraperitoneal and subcutaneous injection with 100 μg of the purified Tromp1-GST fusion protein in a 1:1 mixture of Freund's complete adjuvant. Animals were given booster doses three times at 3-week intervals by intraperitoneal and subcutaneous injections with 75 μg of recombinant Tromp1, which had been cleaved and purified free of the GST moiety, in a 1:1 mixture of incomplete Freund's adjuvant. The titers of the anti-Tromp1 antisera were found to be >1:5,000 when samples were immunoblotted against *T. pallidum* whole-cell lysates. MAb H9-2, an immunoglobulin G1 (IgG1) isotype, was directed against *T. pallidum* FlaA, the periplasmic endoflagellar sheath protein (37). Rabbit antiserum to TpN60, the *T. pallidum* GroEL homolog, was generated against the purified antigen as previously described (35).

SDS-PAGE and immunoblot analysis. Samples were boiled for 5 min in final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% bromophenol blue prior to electrophoresis through 2.4% stacking and 10 or 12.5% separating gels. Gels were then either stained in Coomassie brilliant blue or transferred electrophoretically to a 0.2-μm-pore-size nitrocellulose filter (Schleicher and Schuell, Keene, N.H.) for immunoblotting. Immunoblots were incubated with 1:1,000 dilutions of rat polyclonal Tromp1-GST antiserum or MAb H9-2 followed by sequential incubations with a 1:1,000 dilution of rabbit anti-rat or rabbit anti-mouse IgG-horseradish peroxidase conjugate followed by goat anti-rabbit IgG-horseradish peroxidase conjugate (Zymed, South San Francisco, Calif.). Immunoblots were developed with 4-chloro-1-napthol as the substrate.

Mass spectrometry. One microgram of purified, cleaved recombinant Tromp1 was subjected to electrospray ionization and mass analysis with a Quattro II triple-quadrapole mass spectrometer (Micromass, Altrinchan, England) in the laboratory of Clive A. Slaughter (University of Texas Southwestern Howard Hughes Medical Institute).

Protein sequence analysis. Amino acid sequence analysis of recombinant Tromp1 was performed in the University of Texas Southwestern Medical Center Protein Chemistry Core Facility. Briefly, after SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.). After visualization with Ponceau S, the bands were excised for N-terminal amino acid sequencing by automated Edman degradation (45).

Assessment of porin activity by proteoliposome swelling. The porin activity of Tromp1 was assessed in the laboratory of Hiroshi Nikaido (University of California, Berkeley) by the proteoliposome-swelling assay (20, 46, 49). Graded concentrations of stachyose, an impermeable tetrasaccharide, were used to determine the isosmotic concentration of L-arabinose for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes reconstituted with 0.4 μg of *E. coli* OmpF porin, which was determined to be 180 mM. Influx of L-arabinose into liposomes without protein, proteoliposomes containing 0.4 μg of OmpF, or liposomes reconstituted with 5.4 or 10.8 μg of recombinant Tromp1 was measured as the change in optical density at 600 nm during a 3-min observation period.

CD. Circular dichroism (CD) spectra were obtained on an AVIV (Lakewood, N.J.) model 62DS spectropolarimeter, with sample temperatures being regulated by a Hewlett-Packard model 89100A temperature controller; measurements were taken at 25 ± 0.1°C. CD spectra were obtained on 4 μM samples of Tromp1 or *Neisseria gonorrhoeae* porin P1A (22). Analysis of CD spectra for the recombinant Tromp1 cleaved from the GST moiety was performed with 10 mM phosphate buffer (pH 7.3) with or without 5 mM EDTA and also with 10 mM phosphate buffer containing 0.5% SDS, 0.5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, or 0.6% octylglucoside. CD spectra were obtained for the recombinant gonococcal porin P1A in 10 mM phosphate buffer (pH 7.3) containing 0.6% octylglucoside. All CD spectra were baseline corrected and smoothed with software provided by AVIV Associates. The self-consistent algorithm of Sreerama and Woody (69, 70) was used to calculate the percentages of various types of secondary structures.

Extraction and phase partitioning with Triton X-114. Extraction and phase partitioning with Triton X-114 has been described previously (56, 58). Briefly, freshly harvested *T. pallidum* (10^9), 10 μg of recombinant Tromp1 (cleaved from the GST moiety), and 10 μg of the recombinant gonococcal porin P1A were each added to a 2% solution of Triton X-114 in phosphate-buffered saline (pH 7.4) and rocked overnight at 4°C. Triton X-114-insoluble material was then removed by centrifugation at 20,000 × *g* for 30 min. The resulting supernatants were collected and phase separated, and the detergent-enriched and aqueous phases were washed five times. All samples were then precipitated with 10 volumes of ice-cold acetone for subsequent SDS-PAGE or immunoblot analysis.

Preparation of sucrose gradients. Continuous sucrose density gradients were prepared according to the freeze-thaw procedure described by Baxter-Gabbard (4). A 10% (wt/vol) sucrose solution was subjected to one freeze-thaw cycle by freezing it overnight at −20°C, followed by slow thawing in an ice-water bath. Gradient formation was determined by refractive index measurements. This procedure results in the formation of continuous gradients of 5 to 20% sucrose.

Liposome preparation, sucrose density gradient ultracentrifugation, and vesicle-binding assays. Liposomes consisting of 70 mol% POPC and 30 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were reconstituted with and without protein according to the octylglucoside dilution-dialysis procedure described by Jackson and Litman (39). All samples were prepared in 20 mM HEPES (pH 7.4)–100 mM NaCl–1 mM dithiothreitol–0.5 mM EDTA–0.02% NaN_3. Lipid (2.5 μmol) was initially dried by a slow nitrogen purge followed by lyophilization for at least 3 h. Solid octylglucoside, protein (recombinant Tromp1, bacteriorhodopsin [BR; Sigma Chemical Company, St. Louis, Mo.], or porin P1A), concentrated buffer, and water were then added to yield an initial protein/lipid molar ratio of 1:500 at an octylglucoside concentration of 60 mM in 0.5 ml of buffer. This detergent concentration ensures complete conversion of the sample into mixed micelles (39). Samples were bath sonicated for 1 min and then incubated at 4°C for at least 4 h. The suspension was then diluted in buffer to a final octylglucoside concentration of 10 mM. The vesicles were then dialyzed extensively against buffer, after which they were concentrated with Centricon-30 (Amicon Inc., Beverly, Mass.) ultrafiltration units to about 0.5 ml and loaded onto 5 to 20% sucrose gradients. Vesicles were centrifuged overnight at 150,000 × *g* at 4°C. Lipid and protein gradient profiles were evaluated by phospholipid assay (3) and by Coomassie brilliant blue staining of fractions following SDS-PAGE.

The binding of Tromp1, BR, and P1A to lipid vesicles was assessed by the procedure of Tortorella et al. (74). Vesicles containing 68.9 mol% POPC, 29.5 mol% POPG, and 1.6 mol% biotin-1,2-dihexadecanoly-*sn*-glycero-3-phosphoethanolamine were reconstituted with recombinant Tromp1, BR, or P1A as described above. 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 1:8. Samples were incubated at room temperature for 30 min, followed by centrifugation at 11,000 × *g* for 20 min. The pellet and supernatant fractions were then analyzed by SDS-PAGE and Coomassie brilliant blue staining.

Immunofluorescence analysis of *T. pallidum* encapsulated in gel microdroplets. *T. pallidum* cell suspensions were encapsulated in gel microdroplets as previously described (17). Encapsulated organisms were probed with specific antibodies by a three-step indirect immunofluorescence technique. Briefly, 1:20 dilutions of MAb H9-2, rat anti-Tromp1-GST, and rabbit anti-GroEL were added directly to small aliquots of beads (0.3 to 0.4 ml) in 1 ml of *T. pallidum* cultivation medium (TpCM). In samples incubated with detergent, the appropriate volume of a 1% Triton X-100 (vol/vol) stock solution in phosphate-

buffered saline was added to the beads immediately after the addition of the primary antibody. The samples were incubated with gentle mixing in a 34°C water bath. The beads were washed three times by low-speed centrifugation (100 × *g*) followed by resuspension in 3 ml of TpCM and then a 1-h incubation with biotin-labeled goat anti-rat or goat anti-mouse. Beads were washed as described above, resuspended in 3 ml of TpCM, and incubated with 3 μg of *R*-phycoerythrin coupled to streptavidin (Molecular Probes, Eugene, Ore.). Beads were washed a final time and then viewed on glass slides with a Nikon Optiphot-2 fluorescence microscope equipped with 15× oculars, a dark-field condenser, and a fluorescein filter. Samples were observed with either a 40× or a 100× oil-immersion objective. For each condition (i.e., antibody and detergent concentration) three slides were prepared. Each slide was initially scanned to identify representative fields, and then approximately 100 organisms were scored for labeling (fluorescence). The results from three independent experiments were combined and plotted as means ± standard deviations.

Opsonization assays. Opsonization assays were performed in the laboratory of Sheila A. Lukehart (University of Washington, Seattle) as previously described (1, 2, 44, 66). Values obtained were analyzed by Student's *t* test; *P* values of ≤0.05 were considered significant.

Labeling of *T. pallidum* outer membrane proteins with [^{125}I]TID. Testicular extracts from either uninfected or *T. pallidum*-infected rabbits were cleared of eukaryotic cells and debris by two rounds of centrifugation at 1,000 × *g* for 20 min. The supernatants were then centrifuged at 20,000 × *g* (4°C) for 20 min, and the resulting pellets were resuspended in cross-linking buffer consisting of 0.01 M HEPES, 0.15 M NaCl, and 5 mM $MgCl_2$ (pH 7.5). Fifteen microcuries of 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazarine ([^{125}I]TID) (Amersham) was added to 100 μl of each sample and then photoactivated by exposing the mixtures for 30 min to long-wave UV light (366 nm). Cross-linked samples then were either pelleted or subjected to Triton X-114 phase partitioning and frozen at −70°C for subsequent analyses.

Two-dimensional NEPHGE. Two-dimensional nonequilibrium pH gel electrophoresis (NEPHGE) was performed as previously described (53). First-dimension NEPHGE was performed for 7 h at 400 V in 11-cm tube gels consisting of 4% pH 5 to 7 and 1% pH 3.5 to 10 ampholines (Bio-Rad Laboratories, Hercules, Calif.). Apparent pIs were determined with pI standards (Bio-Rad Laboratories) run under the same conditions.

***T. pallidum* cell fractionation.** Virulent *T. pallidum* organisms were fractionated into outer membranes and cell cylinders by the plasmolysis-based protocol previously described (60). Individual fractions were routinely assayed for NADH oxidase activity and immunoblotted against antibodies specific for Tpp47, FlaA, and GroEL. Transmission electron microscopy of separately pooled outer membranes and cell cylinders was performed to confirm that the outer membrane fractions were devoid of cell cylinders and that outer membranes had been completely removed from the cell cylinders used for the immunoblot studies with anti-Tromp1 antiserum.

Nucleotide sequence analysis. Nucleotide sequencing of the various *tromp1* fusion constructs was performed with an Applied Biosystems Inc. (Foster City, Calif.) model 373A automated DNA sequencer and the PRISM ready-reaction DyeDeoxy Terminator cycle sequencing kit according to the manufacturer's instructions.

Computer analysis. Nucleotide and deduced amino acid sequences were analyzed with the MacVector sequence analysis package version 4.1.1 (International Biotechnologies, Inc., New Haven, Conn.). Phylogenetic trees were constructed with the DISTANCES and GROWTREE programs in the Genetics Computer Group (GCG) version 8.0 software package. Protein similarities and identities were determined with the GAP program from the GCG software package. The *Haemophilus influenzae* Rd adhesin B precursor (FimA) given identification no. HI0362 (26) was used for the analysis shown in Fig. 1.

RESULTS

Tromp1 is homologous to substrate-binding proteins of bacterial ABC transporter complexes. The initial report describing Tromp1 (6) noted that it has sequence similarity to the ScaA family of lipoprotein adhesins identified in gram-positive bacteria, primarily streptococci. Other investigators have reported that *scaA* and its homologs are contiguous to and transcriptionally linked with genes encoding homologs for the integral membrane protein component(s) and ATPases of ABC transporter complexes, suggesting that these adhesins also have transport-related functions (24, 41, 43, 62). Interestingly, more recent database searches revealed that Tromp1 also has a high degree of sequence similarity with periplasmic substrate-binding proteins from ABC transporters of gram-negative bacteria and *Synechocystis* sp. strain 6803, a cyanobacterium with a dual membrane ultrastructure (Fig. 1) (30). Furthermore, global alignment of Tromp1 and its homologs

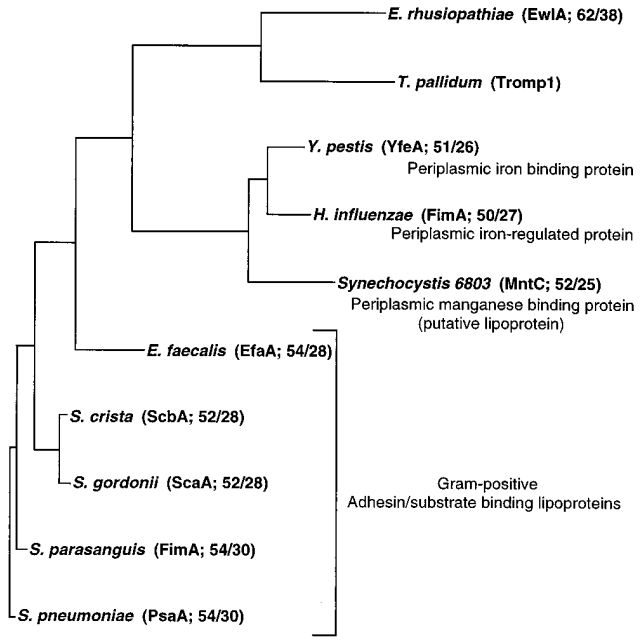

FIG. 1. Evolutionary relationship between Tromp1 and other bacterial adhesin/substrate-binding proteins as determined by phylogenetic analysis with the DISTANCE (Kimura matrix) and GROWTREE algorithms (GCG software package). The BLAST score probabilities between Tromp1 and its homologs range from 1.7×10^{-74} for EwlA to 3.3×10^{-25} for MntC. Percent similarities and identities between Tromp1 and the other proteins are also shown in parentheses alongside each protein designation. *E. rhusiopathiae*, *Erysipelothrix rhusiopathiae*; *Y. pestis*, *Yersinia pestis*; *E. faecalis*, *Enterococcus faecalis*; *S. crista*, *Streptococcus crista*; *S. gordonii*, *Streptococcus gordonii*; *S. parasanguis*, *Streptotoccus parasanguis*; *S. pneumoniae*, *Streptococcus pneumoniae.*

revealed a highly conserved 13-amino-acid motif within the amino-terminal third of each protein (30); highly conserved motifs in other periplasmic substrate-binding proteins have served as signature sequences which have aided in the classification of binding proteins into functionally related groups (73).

Tromp1 contains an uncleaved signal sequence. Outer membrane proteins are synthesized with cleavable N-terminal signal peptides which direct export of the nascent polypeptides across the cytoplasmic membrane (55). Experiments were performed, therefore, to determine whether the hydrophobic stretch at the N terminus of Tromp1 is cleaved.

Our first approach was to evaluate processing of Tromp1 in *E. coli* maxicells (19). Because uncontrolled expression of Tromp1 is toxic in *E. coli* (6), for these experiments we utilized a construct in which the *tromp1* gene was under the control of the bacteriophage T7 promoter. However, expression of the gene was insufficient for pulse-chase labeling experiments. In an attempt to circumvent this problem, the Tromp1 signal sequence plus 11 amino acids of the presumptive mature protein were expressed as a fusion with *E. coli* alkaline phosphatase (PhoA). While this fusion was well expressed, proteolytic degradation precluded interpretable processing experiments.

As an alternative strategy, we compared the SDS-PAGE mobility of native Tromp1 with that of Tromp1 produced in an in vitro-coupled transcription-translation system which lacks signal peptidase I (SPaseI) activity. FlaA, a *T. pallidum* polypeptide which is synthesized with an SPaseI-cleaved leader peptide (38), served as a control. As shown in Fig. 2A, the SDS-PAGE mobilities of the native and in vitro-expressed Tromp1 were identical. In contrast and as expected, the ap-

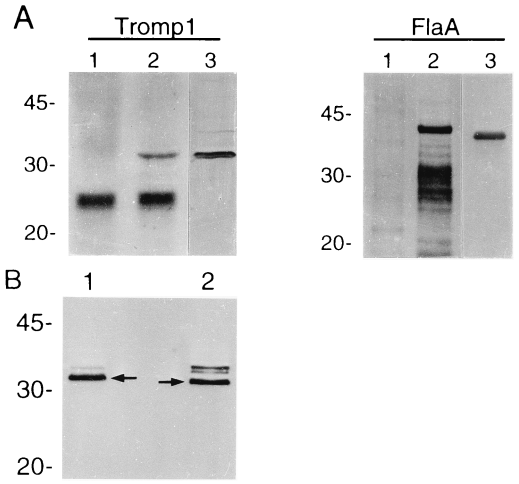

FIG. 2. (A) Native Tromp1 possesses an uncleaved signal sequence. The SDS-PAGE mobilities of Tromp1 and FlaA produced in an in vitro-coupled transcription-translation system (lanes 2) were compared to those of their native counterparts identified by immunoblot analysis with rat anti-Tromp1 antibodies or MAb H9-2 directed against *T. pallidum* FlaA (lanes 3). Lanes 1 contain sham reaction mixtures lacking DNA. Note that the autoradiographs in lanes 1 and 2 and the immunoblots in lanes 3 were obtained from the same nitrocellulose transfer containing samples run in adjacent lanes. (B) Electrophoretic behaviors of recombinant and native Tromp1. *T. pallidum* whole-cell lysates (5×10^8) (lane 1) and recombinant Tromp1 lacking the N-terminal signal sequence (75 ng) (lane 2) were immunoblotted with rat anti-Tromp1 antiserum. The arrows designate the fully denatured forms of the native and recombinant proteins. Molecular mass standards in kilodaltons are shown at the left.

parent molecular mass of the in vitro-expressed FlaA was larger than that of its native counterpart (Fig. 2A). The difference in the apparent molecular masses of the two FlaA proteins (approximately 2,000 Da) correlated well with the deduced size of the FlaA leader peptide (1,930 Da) (38). The results with FlaA indicate that our gel system should have discriminated between processed and unprocessed forms of Tromp1.

Physicochemical properties of Tromp1. Experiments were next conducted to evaluate the physicochemical properties of Tromp1. A major objective of these studies was to determine whether the protein possesses characteristics typically associated with bacterial outer membrane proteins. Given the difficulty in purifying large quantities of native Tromp1 under nondenaturing conditions and without detergents, we relied largely upon the use of a recombinant protein which consisted of the proposed mature (i.e., processed) polypeptide (6) plus two amino acids (G and S) derived from GST (see Materials and Methods). Whereas integral membrane proteins typically are insoluble in aqueous buffers (32), it seemed noteworthy, at the outset, that recombinant Tromp1 was highly soluble in the absence of detergents.

(i) Electrophoretic behavior. Purified recombinant Tromp1 migrated in SDS-polyacrylamide gels as a major polypeptide with an apparent molecular mass of 31,000 Da and a minor component(s) of 34,000 Da (Fig. 2B, lane 2). To avoid potential ambiguity in the interpretation of subsequent studies, we first confirmed that the recombinant protein was homogeneous. Automated Edman degradation revealed that both the major and minor components had the same residues in their N termini (G-S-D-G-K-P), which corresponded to the residues predicted from the nucleotide sequence of the *tromp1-gst* fusion. Additionally, electrospray ionization mass spectrometry revealed that the purified recombinant Tromp1 solution contained a single protein with a molecular mass of 30,581 ± 17 Da (data not shown), a value which is very close to the recombinant protein's calculated molecular mass of 30,603 Da. Thus, we concluded that the purified protein was, indeed, homogeneous and that the species migrating at 31,000 Da is the fully denatured form. When the SDS-PAGE mobilities of native and recombinant Tromp1 were directly compared, two observations were apparent: (i) as with the recombinant protein (Fig. 2B, lane 2), a minor portion of native Tromp1 was not fully denatured (Fig. 2B, lane 1) (note that gels needed to be overloaded with whole-cell lysates to detect this minor form) and (ii) the apparent molecular mass of fully denatured native Tromp1 was 1.5 kDa larger than that of its recombinant counterpart (Fig. 2B). The latter finding is consistent with results of the in vitro transcription-translation studies showing that the native Tromp1 signal sequence is not processed and further supports the fact that the SDS-PAGE conditions utilized here would have resolved a Tromp1 precursor from a processed form of the polypeptide (which is essentially equivalent to the recombinant protein shown in Fig. 2B, lane 2).

(ii) Channel-forming ability. Blanco et al. (7) recently reported that recombinant Tromp1, which had been eluted from polyacrylamide gels with detergents, enhanced channel conductance in black lipid membranes. Therefore, we sought to determine whether recombinant Tromp1 purified in solution under nondenaturing conditions and without detergents possesses porin-like activity. The proteoliposome-swelling assay of Nikaido and coworkers (20, 46, 49) was used to assess the ability of recombinant Tromp1 to form aqueous channels capable of permitting the influx of small, hydrophilic solutes. Transport of the pentose sugar L-arabinose (M_r, 150) was not detected with concentrations of Tromp1 27-fold greater than those used for the *E. coli* OmpF control (data not shown).

(iii) Secondary structure. CD was used to determine whether recombinant Tromp1 contains the extensive β-sheet secondary structure typical of a bacterial outer membrane protein (15, 28, 47, 55). Consistent with the Chou-Fasman (14) and Garnier et al. algorithms (27), which predicted a large amount of alpha-helical structure, the molar ellipticity values in the far UV region (195 to 260 nm) indicated that recombinant Tromp1 was nearly 80% alpha-helix and only 3% β-sheet (Fig. 3 and Table 1). The identical CD spectrum was obtained in the presence of 0.5% SDS, 0.5% CHAPS, or 0.6% octylglucoside (data not shown), indicating that the protein does not undergo a major conformational rearrangement in an amphiphilic environment. Chakrabarti et al. (13) reported that porin OmpU from *Vibrio cholerae* is irreversibly denatured in the presence of EDTA. Although the CD spectrum did not indicate that recombinant Tromp1 was denatured, we analyzed the protein purified without EDTA; a CD spectrum identical to that shown in Fig. 3 was again obtained (data not shown). For comparison purposes, we also analyzed the CD spectrum of the recombinant porin P1A from *N. gonorrhoeae*, which was known to be in native conformation (22). In contrast to recombinant Tromp1, the CD spectrum of the recombinant gonococcal porin showed 45% β-sheet but only 7% alpha-helix (Fig. 3 and Table 1).

(iv) Amphiphilicity. Experiments were next conducted to determine whether recombinant Tromp1 (which corresponds to the presumptive integral outer membrane protein) possesses amphiphilic character. Consistent with its solubility in aqueous solution, recombinant Tromp1 partitioned exclusively into the Triton X-114 aqueous phase (Fig. 4B). By contrast, native Tromp1 partitioned into the detergent-enriched phase, as did the recombinant gonococcal porin (Fig. 4A and C, respectively). As an additional test of amphiphilicity, we exam-

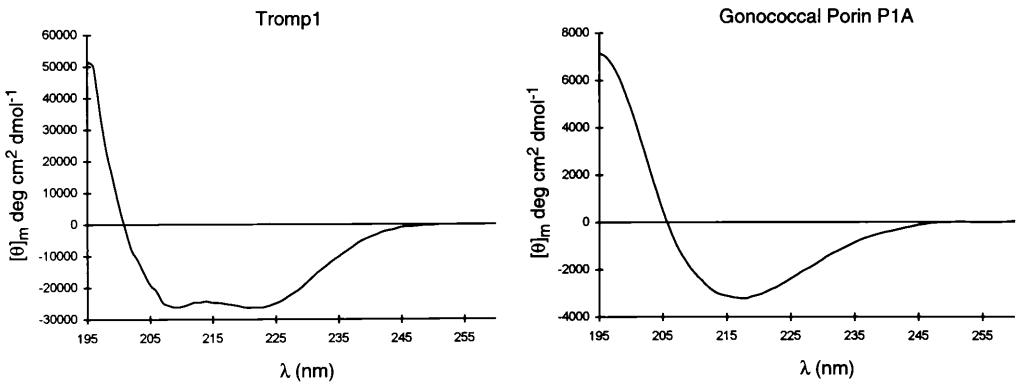

FIG. 3. Secondary structure analysis of Tromp1 by CD spectroscopy. The CD spectrum of recombinant Tromp1 is compared with that of recombinant porin P1A from *N. gonorrhoeae*. The double minimum displayed in the Tromp1 spectrum is typical of proteins with a high percentage of alpha-helix. The single molar ellipticity minimum at 218 nm in the spectrum for porin P1A is typical of proteins with a large percentage of β-sheet. $[\theta]_m$, molar ellipticity.

ined Tromp1's ability to integrate into lipid bilayers. For controls, these experiments utilized BR, a well-characterized integral membrane protein with multiple membrane-spanning domains (29), as well as gonococcal porin P1A. These two proteins, along with recombinant Tromp1, were reconstituted into liposomes composed of a 70:30 mixture of POPC and POPG. After sucrose density gradient ultracentrifugation, fractions from each gradient were analyzed by phospholipid assay and by SDS-PAGE. Liposomes reconstituted with recombinant Tromp1 (Fig. 5B) were no denser than the unloaded control vesicles (Fig. 5A). In contrast, liposomes reconstituted with either BR (Fig. 5C) or the gonococcal porin P1A (Fig. 5D) migrated considerably further into the gradient than did the unloaded control POPC-POPG vesicles (Fig. 5A), indicating that both proteins had integrated into the liposomes. The method of Totorella et al. (74) was employed as an additional means of determining whether Tromp1 will associate with phospholipid bilayers. POPC-POPG liposomes containing small amounts of biotinylated phosphatidylethanolamine were reconstituted in the presence of Tromp1, BR, or P1A, crosslinked with streptavidin, and then centrifuged to separate liposomes from unincorporated protein as described in Materials and Methods. Tromp1 remained exclusively in the supernatant, while both BR and P1A sedimented with the vesicles (data not shown).

Cellular localization of native Tromp1. Four different, though complementary, experimental strategies were employed to establish the location of native Tromp1 within *T. pallidum.*

(i) Immunofluorescence analysis. The first approach involved immunofluorescence analysis of spirochetes encapsulated in agarose beads. This highly sensitive method preserves the integrity of fragile *T. pallidum* outer membranes during surface immunolabeling studies but also enables the detection of intracellular antigens when organisms are coincubated with antibodies and graded concentrations of Triton X-100 (16, 17). Figure 6 shows the combined results for three separate experiments. Intact spirochetes did not fluoresce following incubation with a 1:20 dilution of rat anti-Tromp1 antiserum. By contrast, 43% of the spirochetes were labeled by anti-Tromp1 antibodies when they were incubated with 0.06% Triton X-100, a detergent concentration which selectively exposes FlaA, the periplasmic marker. At higher concentrations of detergent, labeling with the anti-Tromp1 antibodies paralleled the labeling obtained with antibodies specific for TpN60, a GroEL homolog which is distributed between the cytoplasmic membrane and cytoplasm (64); with both antigens, 100% labeling was obtained with 0.15% Triton X-100. Anti-GST antiserum failed to react with either intact or detergent-treated organisms (data not shown).

(ii) Opsonization by anti-Tromp1 antiserum. Opsonization of virulent treponemes by immune rabbit serum (IRS) is believed to reflect the binding of antibodies to surface-exposed domains of rare outer membrane proteins (10, 57). Thus, we also compared the abilities of rabbit anti-Tromp1 antiserum and IRS to promote phagocytosis of motile *T. pallidum* by rabbit peritoneal macrophages (1, 2, 44, 66). In three separate experiments conducted in a blind manner, no increase in opsonization was observed with the anti-Tromp1 antiserum (Table 2).

TABLE 1. Secondary structure percentages calculated from CD spectra

Protein	% of secondary structure[a]:			
	Alpha-helix	β-sheet	Turn	Other
Tromp1	77	3	9	11
Porin P1A	7	45	19	29

[a] Percentages were determined from the molar ellipticities calculated from the CD spectra with the self-consistent algorithm of Sreerama and Woody (69, 70).

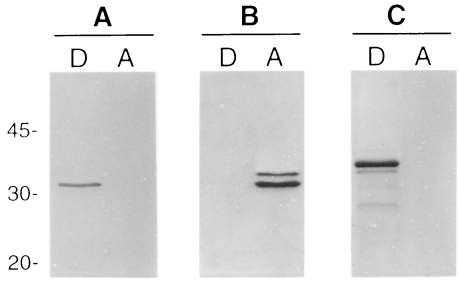

FIG. 4. Triton X-114 phase partitioning of native and recombinant Tromp1. *T. pallidum* (5×10^7) (A), recombinant Tromp1 (200 ng) (B), and recombinant gonococcal porin P1A (200 ng) (C) were subjected to Triton X-114 phase partitioning. Detergent-enriched (lanes D) and aqueous (lanes A) phases were separated by SDS-PAGE followed by either immunoblot analysis with rat anti-Tromp1 antiserum (A) or staining with Coomassie brilliant blue (B and C). Shown at the left are molecular mass standards in kilodaltons.

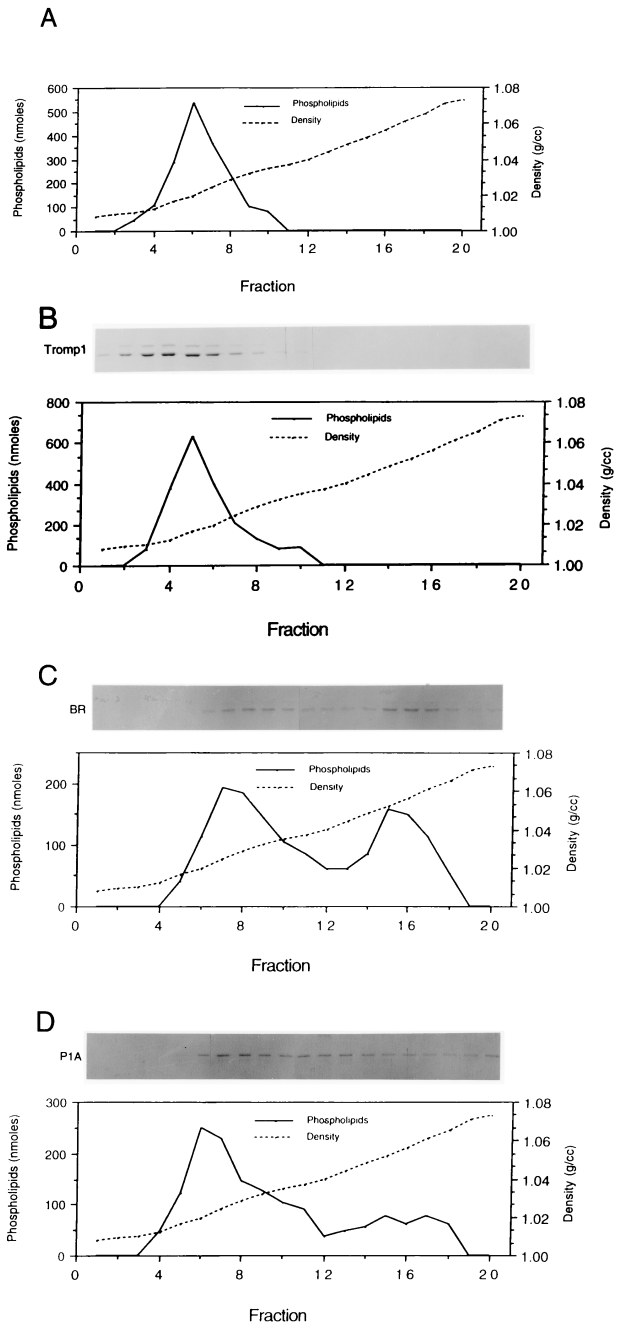

FIG. 5. Inability of recombinant Tromp1 to integrate into phospholipid vesicles. Liposomes containing a 70:30 mixture of POPC and POPG were reconstituted without protein (A) or with recombinant Tromp1 (B), BR (C), or gonococcal porin P1A (D) followed by sucrose density gradient centrifugation. The fractions from each gradient were then assayed for phospholipids and analyzed by SDS-PAGE.

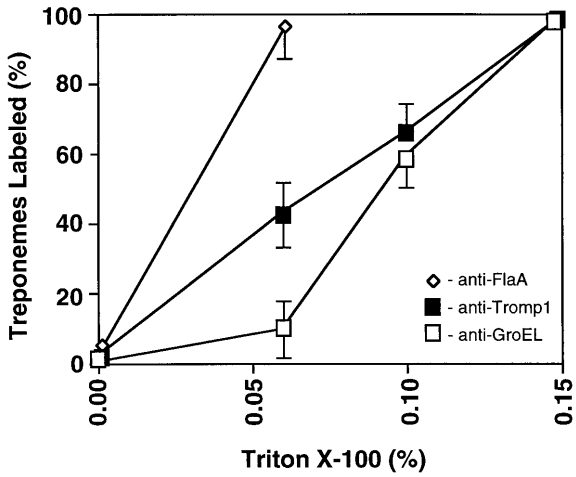

FIG. 6. Localization of Tromp1 in *T. pallidum* encapsulated in gel microdroplets. Freshly encapsulated treponemes were probed with antibodies directed against Tromp1, FlaA (periplasmic marker), and GroEL (cytoplasmic membrane and cytoplasmic marker) in the presence of graded concentrations of Triton X-100. Labeling was scored by comparison with results of darkfield microscopy. Each point represents the mean ± standard deviation of three determinations (approximately 100 organisms for each determination) from each of three separate experiments.

(iii) Radiolabeling of *T. pallidum* outer membranes with a photoactivatable, lipophilic probe. An antibody-independent method also was used to detect Tromp1 within the outer membranes of intact treponemes. [^{125}I]TID is a photoactivatable lipophilic reagent which has been used extensively to label the hydrophobic domains of proteins within the apolar cores of membranes (12, 23, 34). This extremely hydrophobic reagent partitions into lipid bilayers and, upon exposure to UV light, becomes cross-linked to the fatty acid chains of lipids and the side chains of amino acids. The carbene generated by photoactivation of [^{125}I]TID reacts with alkyl groups and is not restricted to nucleophiles such as amino and sulfhydryl moieties; consequently, any amino acid within a hydrophobic environment can be labeled. Because Tromp1 was predicted to possess 14 membrane-spanning domains (6), it seemed reasonable to expect it to be radiolabeled. We also reasoned that labeling of intact treponemes would be outer membrane-specific because the outer membranes would trap the probe and prevent its ingress into subsurface compartments. [^{125}I]TID labeled a single amphiphilic protein (Fig. 7A, lane 2) whose mobility by SDS-PAGE (apparent molecular mass of 31,000 Da) was slightly faster than Tromp1's (Fig. 7A, lane 1); interestingly, this protein also happened to comigrate with a previously identified candidate rare outer membrane protein (compare Fig. 7A, lane 1, with Fig. 8, lane SS). Two-dimensional NEPHGE analysis of the Triton X-114 detergent-enriched phase from *T. pallidum* verified that native Tromp1 (calculated pI of 6.6) and the radiolabeled polypeptide (apparent pI of 8.8) were distinct, amphiphilic proteins (Fig. 7B). No radiolabeled proteins were detected in the Triton X-114 extracts from iden-

TABLE 2. Inability of anti-Tromp1 antiserum to promote macrophage opsonization of *T. pallidum*

Serum tested	Mean % of macrophages ingesting *T. pallidum* ± SD[b]	*P* value			
		IRS	Tromp1	GST	NRS
IRS	41 ± 6		0.004	0.01	0.01
Tromp1	19 ± 2			NS[c]	NS
GST	23 ± 3				NS
NRS[a]	23 ± 3				

[a] NRS, normal rabbit serum.
[b] Mean percentage ± standard deviation values were calculated from three replicates.
[c] NS, not significant.

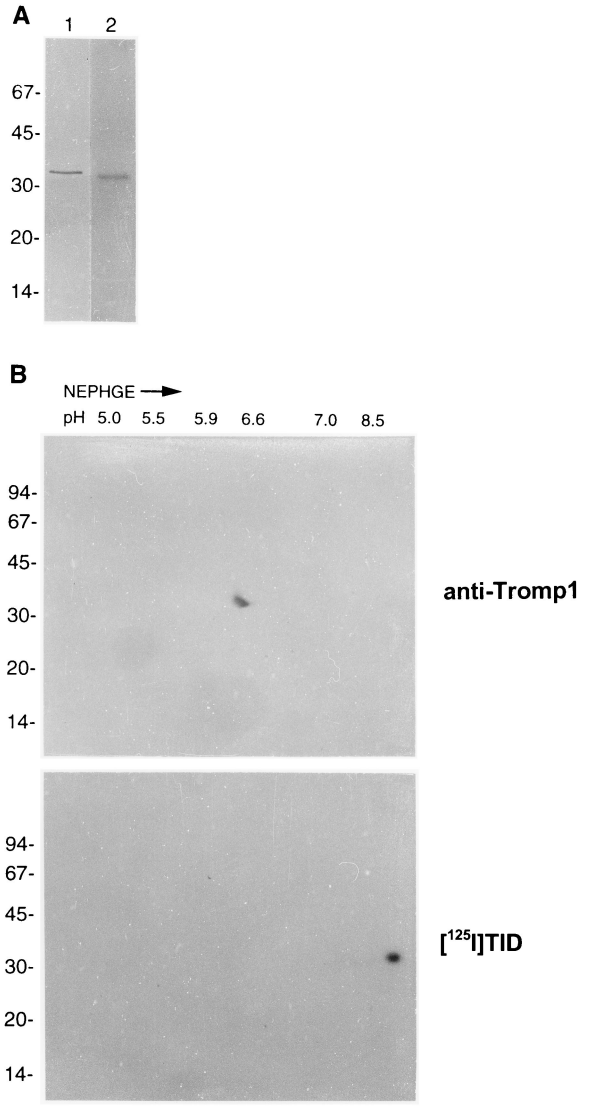

FIG. 7. Identification of a potential *T. pallidum* rare outer membrane protein by radiolabeling with [^{125}I]TID. (A) The Triton X-114 detergent-enriched phase from motile treponemes incubated with the lipophilic, photoactivatable probe [^{125}I]TID was subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose strip was first immunoblotted with rat anti-Tromp1 antiserum (lane 1) and then subjected to autoradiography (lane 2). (B) The same material as in panel A was analyzed by two-dimensional NEPHGE. Following two-dimensional NEPHGE and transfer to a nitrocellulose filter, the filter was first immunoblotted (anti-Tromp1) and then subjected to autoradiography ([^{125}I]TID) as described above. Molecular mass standards in kilodaltons are shown at the left. pH markers are shown above the NEPHGE gels.

tically treated normal rabbit testicular suspensions (data not shown).

(iv) Cell fractionation. Finally, the cellular distribution of Tromp1 was examined by a gentle, plasmolysis-based protocol which separates *T. pallidum* into outer membranes and cell cylinders (which contain the PG-CM complexes) (60). Immunoblot analysis confirmed that Tromp1 was present in isolated outer membranes (Fig. 8, lanes SS and A). However, outer membranes from 1.5×10^9 organisms were needed to obtain an immunoblot signal of an intensity approximately equal to that obtained from 3×10^7 cell cylinders (Fig. 8, lanes A and B). The observation that cell cylinders and whole-cell lysates contained approximately equal amounts of the protein (Fig. 8, lanes B and C) also indicated that relatively small amounts of Tromp1 were recovered in the outer membrane fractions. During our previous characterization of isolated *T. pallidum* outer membranes (60), we designated several polypeptides as candidate rare outer membrane proteins because they appeared to be highly enriched in isolated outer membranes with respect to cell cylinders and whole cells (60). At the time when these candidates were first identified, it was not known whether Tromp1 was in the outer membrane preparations. Combined silver staining and immunoblot analysis of outer membranes revealed that Tromp1 was not a member of this subset of outer membrane-associated proteins (Fig. 8, lane SS).

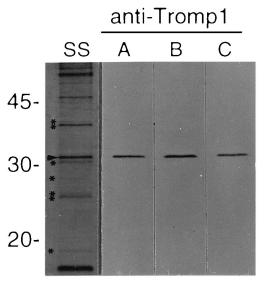

FIG. 8. Tromp1 is predominantly associated with *T. pallidum* cell cylinders. The lane designated SS is a silver stain of outer membranes isolated from 3×10^9 treponemes; the arrowhead designates Tromp1, while the asterisks designate previously identified candidate rare outer membrane proteins (60). Lanes A, B, and C contain, respectively, outer membranes from 1.5×10^9 treponemes, cell cylinders from 3×10^7 treponemes, and 3×10^7 whole-cell lysates immunoblotted with the same dilution (1:500) of rat anti-Tromp1 antiserum. Molecular mass standards in kilodaltons are shown to the left of the SS lane.

DISCUSSION

The development of techniques for isolating *T. pallidum* outer membranes has been a significant advance for efforts to characterize rare outer membrane proteins (57). Nevertheless, there are two principal reasons why utilization of isolated outer membranes for this purpose has been less straightforward than originally anticipated. First, because of the lack of a priori knowledge of *T. pallidum* outer membrane constituents and the paucity of monospecific antibodies directed against treponemal outer membrane proteins, it has not been possible to directly correlate intramembranous particles visualized by freeze-fracture electron microscopy with individual outer membrane-associated proteins visualized by SDS-PAGE. Second, because isolated outer membranes contain subsurface contaminants (9, 60, 67), the mere presence of a previously uncharacterized polypeptide in isolated outer membranes cannot be considered tantamount to its being a rare outer membrane protein. These caveats have necessitated the development of an algorithm to guard against prematurely designating polypeptides in isolated outer membranes as rare outer membrane proteins (57, 67). The first step involves designation of highly enriched outer membrane-associated polypeptides as candidate outer membrane proteins. This is followed by molecular cloning studies to determine whether a candidate possesses properties typical of gram-negative bacterial outer membrane proteins (57). The final step is obtaining definitive evidence for outer membrane location and surface exposure in intact treponemes.

The starting point for our study was the observation that Tromp1 has highly significant sequence relatedness to the

ScaA family of gram-positive bacterial lipoprotein adhesins (6, 7, 30). At the outset, it seemed peculiar that an outer membrane protein is evolutionarily related to surface-exposed lipoproteins from organisms which lack outer membranes. Furthermore, because the polypeptide portions of bacterial lipoproteins are hydrophilic while outer membrane proteins, including porins, are folded predominantly into amphiphilic β-pleated sheet structures (15, 28, 47, 55, 63), it appeared unlikely that proteins with extensive primary sequence similarity can adopt such different secondary structures. Consistent with this reasoning, the more recently identified Tromp1 homologs from gram-negative bacteria are periplasmic substrate-binding proteins. Underscoring the relevance of these genetic analyses is the finding that the *tromp1* gene, like each of its homologs, is transcriptionally linked to open reading frames which encode additional ABC transporter complex components which, in gram-negative bacteria, are integral cytoplasmic membrane proteins (i.e., permeases) or cytoplasmic membrane-associated ATPases (30, 33).

Although Blanco and coworkers proposed that the nascent Tromp1 polypeptide possesses a cleaved signal peptide, they based this contention on evidence obtained using a construct in which the putative processed form of Tromp1 was fused to the *E. coli* OmpT leader peptide (6). Our demonstration that Tromp1 produced in an in vitro-coupled transcription-translation reaction lacking SPaseI activity was identical in size to the native protein is inconsistent with cleavage of the Tromp1 N-terminal signal sequence. The larger molecular mass of native Tromp1, compared to that of the recombinant, further supports this contention. The 5 amino acids preceding the proposed cleavage site of Tromp1 are K-D-A-A-A (6). SPaseI cleavage sites rarely contain three alanine residues, while the amino acids immediately preceding the cleavage site typically are polar, not charged (75, 76). The inability of SPaseI to process the Tromp1 signal sequence is likely due to these deviations from the consensus (21, 55). In any event, Tromp1 is not expected to translocate through the *T. pallidum* cytoplasmic membrane and localize in the outer membrane with an uncleaved signal sequence (55).

At the primary sequence level, outer membrane proteins of gram-negative bacteria possess an overall hydrophobicity similar to that of periplasmic proteins, and it has been presumed that this situation also pertains to *T. pallidum* despite the markedly different compositions of treponemal and gram-negative bacterial outer membranes (57). Because proteins destined for the periplasmic space and outer membranes cannot be distinguished easily with existing computer algorithms (28, 63), it must be determined empirically whether a candidate *T. pallidum* polypeptide has the secondary structure and amphiphilicity characteristic of an outer membrane protein. According to the topology proposed by Blanco et al. (6), Tromp1 should have contained approximately 45% β-sheet structure. Using CD, however, we found that an undenatured recombinant form of Tromp1 lacking its N-terminal signal sequence consisted predominantly (77%) of alpha-helix and possessed little (3%) β-sheet secondary structure. This contrasted sharply with the CD spectrum for a recombinant gonococcal porin, which did contain extensive (45%) β-sheet structure. Furthermore, several lines of evidence demonstrated that recombinant Tromp1, in contrast to the recombinant gonococcal porin P1A, is hydrophilic and exhibited virtually no propensity to associate with bilayers composed of two phospholipids which are well-represented in *T. pallidum* outer membranes (60). Unlike recombinant Tromp1, native Tromp1 is amphiphilic. A hydrophilic protein, such as *E. coli* alkaline phosphatase, will become amphiphilic when it is fused to an uncleaved signal sequence (8). Therefore, the simplest explanation for the markedly different solubility properties of native and recombinant Tromp1 is that the uncleaved signal sequence confers amphiphilicity on the remainder of the protein.

In gram-negative bacteria, outer membrane proteins are exported as periplasmic intermediates which assume their native conformations and trimeric states during integration into the outer membrane (55, 65, 71). However, we saw no evidence by CD spectroscopy or liposome incorporation for changes in either conformation or amphiphilicity which indicates that the soluble recombinant form of Tromp1 studied here represents an analogous intermediate. Knowing that Tromp1 consists of a hydrophilic protein with a hydrophobic N-terminal domain enables one to predict its membrane topology; such proteins typically are periplasmic, with the uncleaved signal sequence serving as a cytoplasmic membrane anchor. To our knowledge, this is the first example of a substrate-binding protein anchored to a cytoplasmic membrane by an uncleaved signal sequence. Interestingly, a single base substitution (G to T at base 56 in the Tromp1 open reading frame) changes the phenylalanine at residue 19 to a cysteine (30), thereby creating an appropriately placed consensus lipoprotein modification motif (L-T-G-C) (31). It is tempting to speculate, therefore, that the uncleaved signal sequence resulted from a point mutation in the "lipobox" of the ancestral *tromp1* gene and that this mutation was retained because it did not affect the function or membrane topology of Tromp1. Support for this notion is provided by the finding that a mutant maltose-binding protein anchored to the *E. coli* cytoplasmic membrane by an uncleaved signal sequence is fully functional (25).

Porins are defined physiologically by their ability to form aqueous channels which facilitate the passive diffusion of small hydrophilic solutes across bacterial membranes (47). The principle underlying the proteoliposome-swelling assay developed by Nikaido and coworkers is that a putative porin must be capable of inserting into a lipid bilayer and form a channel large enough to permit the influx of solute in the presence of an osmotic gradient (47). A negative result might be due to a protein's inability to integrate into the vesicles, the protein's inability to form functional (or open) channels after integrating into the bilayer, or the use of a solute which is too large to penetrate the aqueous channels formed by the porin. Given that bacterial porins typically have exclusion limits of at least 600 Da (47), the last possibility was excluded by using the pentose sugar L-arabinose (M_r, 150); based upon its calculated pore size(s) (6, 7), arabinose should have readily penetrated Tromp1 channels. The most plausible explanation, therefore, for the lack of liposomal swelling activity is that the hydrophilic recombinant protein failed to integrate into the liposomes. How can these findings be reconciled with results of studies showing that native and recombinant Tromp1 increased conductance across black lipid membranes? There are at least two possible explanations for the increased channel conductance reported by Blanco et al. (6, 7). First, these studies (6, 7) employed proteins which were eluted from polyacrylamide gels with Triton X-100, a detergent known to form ion-conducting channels in black lipid membranes (61). Second, black lipid membrane conductance assays measure individual insertional events and, therefore, may reflect the presence of a minor contaminant(s) capable of forming pores (42). We also should note that the conductance assays performed by Blanco et al. (6, 7) did not include either similarly purified nonporin proteins or recombinant Tromp1 purified under nondenaturing conditions and produced free of detergents as was done in our study.

Tromp1 localization experiments with intact *T. pallidum* were not previously reported. Using several lines of experi-

mentation, we were unable to detect Tromp1 either on the surfaces or within the outer membranes of intact organisms. It might conceivably be argued that the lack of surface localization reflected the absence of antibodies in the anti-Tromp1 antisera directed against key, surface-exposed conformational epitopes; however, this concern is negated by the observation from the immunofluorescence studies that the antisera recognized the native antigen under nondenaturing conditions once the outer membranes were removed with relatively low concentrations of the nonionic detergent Triton X-100. The immunofluorescence experiments further demonstrated that Tromp1 appears to be almost equally distributed between the periplasmic space and a deeper location within the cell, which, given the native protein's amphiphilicity and the topological considerations discussed earlier, is almost certainly within the PG-CM complex. It is interesting that in the gel microdroplet assay, the *T. pallidum* homolog for MglB, a lipid-modified substrate-binding protein (5), appears to be similarly distributed between the periplasmic space and the PG-CM complex (18). To complement these antibody-based approaches, we also labeled intact treponemes with a photoactivatable lipophilic probe, [^{125}I]TID. The outer membrane specificity of the labeling procedure was indicated by the finding that no other *T. pallidum* proteins, including subsurface treponemal proteins (e.g., FlaA and Tpp47) which are known to be radiolabeled by conventional surface iodination (40, 51, 72), were labeled by [^{125}I]TID. Finally, fractionation experiments showed that the preponderance of Tromp1 was associated with cell cylinders. Thus, rather than being enriched in outer membranes, the above-noted data lead us to conclude that the outer membrane-associated portion of Tromp1 represents contamination from a subsurface compartment.

There are two distinct, but interrelated, outcomes from the experiments described here. The first is the accumulation of a cohesive body of evidence which supports the proposal by Hardham et al. (30) that Tromp1 subserves a transport-related function with respect to the cytoplasmic, not the outer, membrane of *T. pallidum*. It is tempting to speculate that this function involves heavy metals, possibly iron, based upon the following observations (30): (i) the Tromp1 homologs in dual-membrane organisms either bind heavy metals or are iron regulated (Fig. 1), (ii) other potential transporter components within the Tromp1 operon are homologous to ABC transporter components involved in iron transport in gram-negative bacteria, (iii) the operon encoding Tromp1 also encodes a homolog for DtxR, an iron-regulated transcriptional repressor in *Corynebacterium diphtheriae*, and (iv) a potential stem-loop structure which resembles a Fur-binding site overlaps the promoter region of this operon.

The second major outcome has been the identification by [^{125}I]TID radiolabeling of an amphiphilic protein which appears to comigrate by SDS-PAGE with a previously designated candidate outer membrane protein. Based upon its molecular mass and the fact that it can be visualized only by two-dimensional NEPHGE, we believe that this is indeed a novel *T. pallidum* membrane protein (50, 52). In two prior freeze-fracture analyses (11, 59), we noted the size homogeneity of outer membrane particles and proposed that there may be a rather limited number of proteins within the *T. pallidum* outer membrane, perhaps only a single protein species. Our results with [^{125}I]TID further argue against the existence of numerous, distinct outer membrane proteins in *T. pallidum*. By enabling us to circumvent laborious molecular cloning and characterization of candidate polypeptides which may not be bona fide outer membrane-spanning proteins, the use of [^{125}I]TID appears to be a potential breakthrough in the ongoing search for *T. pallidum* rare outer membrane proteins.

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