The nature of the surface of the spirochete Treponema pallidum subsp. pallidum (T. pallidum), the causative agent of syphilis, remains enigmatic. Recent investigations indicate that the outer membrane of T. pallidum differs from those of gram-negative bacteria, especially from that of Escherichia coli, in significant features: the T. pallidum outer membrane lacks typical lipopolysaccharide (33), is sensitive to routine laboratory manipulations such as centrifugation and resuspension (31, 32), requires postfixation with uranyl acetate to be visible in thin-section electron microscopy (29), and appears to be removed easily by low concentrations of both ionic and nonionic detergents (28, 31, 41). Attempts to fractionate T. pallidum (28, 31, 41) have demonstrated that the minimal concentration of detergent sufficient to remove the outer membrane does not extract significant quantities of protein into the putative outer membrane fraction. The combined results are consistent with a hypothesis that the treponemal outer membrane may have a comparatively low protein content (28, 31, 41). Recent freeze-fracture electron microscopy has provided ultrastructural evidence for this hypothesis (34).

Recently, genes encoding treponemal polypeptides have been cloned and expressed in E. coli (3, 8, 11, 12, 14, 16, 17, 24, 28, 42-44, 46). Many of these proteins are purportedly either surface-associated or membrane proteins of T. pallidum (3, 8, 11, 14, 16, 24, 32, 43). The putative locations of the native antigen were assigned on the basis of extrinsic lactoperoxidase-catalyzed iodination (18, 24, 43), by localization of the recombinant antigen in the cell envelope fractions of E. coli (3, 11, 14), and by the reactivity of monoclonal antibodies (MAbs) or monospecific antiserum prepared against the E. coli-derived recombinant antigens in immunofluorescence assays of fixed T. pallidum (14), in surface immunoelectron microscopy of T. pallidum (11, 16, 22, 32), in the in vitro-in vivo neutralization assay (18), and in the T. pallidum immobilization test (11, 12, 18, 22). Some of the conclusions derived from these approaches are inconsistent with the hypothesis that the T. pallidum outer membrane contains a relative paucity of proteins. The inconsistency probably arises because the techniques that are used to localize the corresponding treponemal antigen are indirect and require the use of membrane perturbants, membrane-damaging techniques, or extended incubations in the presence of complement.

Swancutt et al. (43) reported the isolation of E. coli clones expressing a 34-kilodalton (kDa) antigen of T. pallidum by screening an E. coli clone bank with specific anti-T. pallidum MAbs; the antigen possesses a unique and characteristic heterogeneous electrophoretic mobility (24, 26, 43). Because extrinsically added lactoperoxidase and radioactive iodine labeled this antigen (24, 43), it was presumed to have a surface-associated location in T. pallidum. The detection of this molecule by a monospecific antisera in immunoelectron microscopy of methanol-fixed whole T. pallidum (16) and the reported abrogation of antibody binding by prior extraction of T. pallidum with Triton X-100 (16, 28) suggest that the 34-kDa antigen is located within the cell envelope of T. pallidum. Neither of these approaches, however, demonstrates with certainty that this antigen is located in the outer membrane. Recently, Radolf et al. (31, 33) have demonstrated that the 34-kDa antigen segregates into a fraction rich in membrane proteins after extraction of intact T. pallidum with Triton X-114, indicating that the 34-kDa antigen behaves biochemically as an integral membrane protein. The combined data suggest that the 34-kDa antigen is probably a constituent of the T. pallidum cell envelope.

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Despite the problems inherent in defining the precise location of individual antigens, determination of the structure of individual T. pallidum proteins, especially cell envelope proteins, will be necessary for an increased understanding of the biology of the organism. Therefore, we determined the DNA sequence of the gene encoding the 34-kDa antigen in an attempt to elucidate the structural and biochemical features of this immunogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. T. pallidum subsp. pallidum (Nichols strain) was cultivated by serial intratesticular passage in New Zealand White rabbits (18). E. coli RR1 (F' rpsL20 lysA4 proA2 lacY1 galK2 rpsL20 [Sm'] xyl-5 mtl-l supE44 ë') was used for plasmid maintenance. E. coli ORN103 (27) was used for minicell analysis. E. coli JM101 [trpC:: Tn10 Δ(lac proAB) thi supE (F' traD36 proA+ proB+ lacIq lacZΔM15)] was used as the cloning host in the propagation of recombinant M13 derivatives for DNA sequence analysis (23). The recombinant plasmid pMN20, which encodes the 34-kDa treponemal antigen, was described previously (43). Recombinant plasmid pNC81 expresses the 47-kDa antigen of T. pallidum (3). Plasmid DNA was prepared by a standard method (43). M13mp19 (25) was used as the phage cloning vector for DNA sequence determination. JM101 and M13mp19 were purchased from New England Biolabs, Inc. (Beverly, Mass.).

Antisera and MAbs. MAbs were generated, maintained, and purified as previously described (35). MAb 11E3 (IgG2b) is specific for the T. pallidum 47-kDa integral membrane protein (3, 18, 24). MAb 9B12 (IgG1), directed against the 34-kDa antigen of T. pallidum, was described previously (43). MAbs 5C6 (IgG2b), 5C11 (IgG2b), 3G9 (IgG2b), and 7A7 (IgG1), also specific for the 34-kDa antigen, were isolated (35) by using Triton X-114-extractable detergent-phase proteins of T. pallidum (31, 33) as immunizing antigens in BALB/c mice. Rabbit antiserum to T. pallidum detergent-phase proteins was described previously (31, 33). Horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G (IgG), goat anti-mouse IgG, and goat anti-rabbit IgG, were purchased from Organon Teknika-Cappel (Malvern, Pa.).

Enzymes and chemicals. Triton X-114 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). N-Laurylsarcosine (sarcosyl) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sequence was obtained from the United States Biological Corp. (Cleveland, Ohio). L-[35S]methionine was obtained from Amersham Corp. (Arlington Heights, Ill.). [α-32P]dATP and [14C]-protein molecular weight markers were purchased from Dupont, NEN Research Products (Boston, Mass.). The BCA protein assay was obtained from Pierce Chemical Co. (Rockford, Ill.). Prestained Rainbow molecular weight markers were purchased from Amersham.

Subcloning of the pMN20 insert and DNA sequence analyses. For DNA sequence determination (37), DNA restriction fragments from pMN20 were subcloned into M13mp19 in E. coli JM101 by standard methods (21). Sequence reactions were performed using Klenow polymerase according to the New England Biolabs sequencing kit. All clones were also sequenced by using the chemically modified T7 DNA polymerase (Sequenase) according to the instructions of the manufacturer. The DNA sequence of both strands of the pMN20 insert was determined completely. [α-32P]dATP was used as the radioactive nucleotide. Sequence reaction mixtures were electrophoresed on 7.5% polyacrylamide gels containing 8.3 M urea in Tris-borate–EDTA buffer (21); the dried gels were subjected to autoradiography, using Kodak X-OMat AR film.

Determination of the amino acid sequence of the 34-kDa antigen. The 34-kDa antigen was partially purified from Triton X-114 detergent-phase extracts of T. pallidum by using affinity columns generated by conjugating MAbs 5C6, 9B12, and 7A7 to Reactigel 6X (Pierce Chemical Co.) as described previously (3, 17). The enriched antigen preparation was electrophoresed through a sodium dodecyl sulfate (SDS)-polyacrylamide gel and electroblotted (17) to polyvinylidene difluoride membranes (Millipore Corp. Bedford, Mass.). (17). The 34-kDa antigen was identified in the appropriate region of the filter by staining the membrane with the reversible stain Ponceau S (17). The portion of the membrane containing the 34-kDa antigen was treated with cyanogen bromide (17). N-Terminal amino acid sequencing of the mixed polypeptides was performed as described previously (17).

Extraction of T. pallidum and E. coli with Triton X-114. Extraction of T. pallidum with 2% Triton X-114 for the isolation of detergent-phase proteins was described previously (31, 33). E. coli RR1(pMN20), expressing the 34-kDa antigen, was grown in 100 ml of LB (21) to an optical density at 550 nm of 0.5 (approximately 3 × 10⁸ CFU/ml). The culture was subjected to centrifugation at 4,000 × g for 10 min at 4°C, and the supernatant was discarded. The pellet was washed once by centrifugation in 1 volume of 200 mM Tris hydrochloride (pH 8.0), and the cell pellet was suspended in 1 ml of 20 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–2% Triton X-114. After incubation for 4 h at 4°C, cellular debris was removed by centrifugation. The supernatant was removed and warmed to 37°C in a water bath to allow phase separation to occur. The separated aqueous and detergent phases were washed in a manner identical to that described previously for T. pallidum (31).

Cell fractionation of recombinant E. coli expressing the 34-kDa antigen. Membranes were prepared by the method of Filip et al. (13) essentially as modified by Chamberlain et al. (3). E. coli RR1 containing pMN20 was grown overnight in LB containing 12.5 μg of chloramphenicol per ml. Cells were harvested by centrifugation at 4,000 × g for 20 min at 4°C. The cells were suspended in 1/10 volume of 200 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and washed once by centrifugation. The cell pellet was suspended in 1/50 volume of 200 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–10% sucrose. The cell suspension was frozen and thawed twice followed by the addition of lysozyme to a final concentration of 200 μg/ml. After incubation on ice for 45 min, the cells were again subjected to two freeze-thaw cycles and then sonicated at 0°C for 5 min by using a Branson sonicator and a microtip sonating probe (50% duty cycle at a power setting of 7). The total lysate was centrifuged at 8,000 × g for 20 min at 4°C to pellet unlysed cells. The supernatant was centrifuged at 110,000 × g for 90 min at 4°C in a Beckman 50.2 Ti rotor. The supernatant (soluble fraction) was removed. The crude cell envelope pellet was washed by suspension in 20 mM Tris hydrochloride (pH 8.0)–0.5 M NaCl, followed by centrifugation at 110,000 × g for 90 min at 4°C. The washed cell envelope pellet was suspended in 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.4) at a protein concentration of approximately 20 mg/ml. An equal volume of 4% sarcosyl in 20 mM HEPES (pH 7.4) was added to the cell envelope preparation and incubated for 1 h at 4°C for a final protein concentration of 10 mg/ml; insoluble material was
collected by centrifugation at 110,000 × g for 90 min (4°C) in a Beckman 70.1 Ti rotor. Cell envelopes, at a concentration of about 2 mg/ml, were also extracted in 2% Triton X-100, as described by Schnaitman (38, 39). MgCl₂ and trisodium EDTA were each used at a concentration of 10 mM. For each detergent extraction, the insoluble pellets were suspended in 20 mM HEPES (pH 7.4) at the original extraction volume. The proteins from both detergent-soluble and detergent-insoluble fractions were precipitated by the addition of 10 volumes of acetone to each fraction and storage at −20°C overnight. Following centrifugation at 30,000 × g, the precipitate of each fraction was suspended in 1% SDS-20 mM HEPES (pH 7.4) to the same volume as the original extraction mixture. Equal volumes of the two fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Prokaryotic DNA-directed in vitro translation assay. An E. coli-based cell-free translation assay system was obtained from Amersham and used according to the instructions of the manufacturer. Each reaction mixture contained 5 µg of plasmid DNA and 30 µCi of L-[^35]S]methionine (>800 Ci/mmol). The reaction mixtures were incubated for 60 min and chased for 5 min at 37°C. After completion, 2 × 10⁷ T. pallidum organisms were added to each sample, and the mixture was diluted with sample buffer (22). After boiling and reduction, the samples were processed for SDS-PAGE and immunoblotting. The nitrocellulose filter was subjected to autoradiography, using Fuji X-ray film and Dupont Cronex Lightning-Plus intensifying screens.

Minicell preparation. Minicells were prepared by a modification of the sucrose density gradient method (2). E. coli ORN103 minA minB(pMN20) was grown overnight at 37°C in 1 liter of LB containing 12.5 µg of chlorotetracycline per ml. The cells were centrifuged at 4,000 × g for 15 min at 4°C; the cell pellet was suspended in 12 ml of LB and layered on top of four sucrose gradients. The sucrose gradients were formed by dissolving 45 g of sucrose in 150 ml of M9 minimal medium (21), dispensing 24 ml in 30-ml centrifuge tubes, freezing completely at −70°C, and slowly thawing overnight at 4°C. Three milliliters of cell suspension was layered onto each gradient. The gradients were centrifuged at 4,000 × g for 20 min at 4°C. The upper band, enriched in minicells, was removed and was centrifuged at 3,000 × g for 15 min at 4°C. The supernatant was discarded. The minicell pellet was resuspended in 2 ml of LB, and 1 ml was applied to each of two additional sucrose gradients. The minicells were then centrifuged as described above, washed once in M9, suspended in M9–20% glycerol at an A₆₀₀ of 2.0, and frozen in aliquots at −70°C until further use.

Expression in minicells. For expression experiments, aliquots of minicells were thawed and washed once by centrifugation at 13,000 × g for 2 min in 1 ml of M9 medium–0.2% glucose–20 µg of d-cycloserine per ml. The minicells were then suspended in M9 containing glucose and cycloserine. They were then preincubated at 37°C for 15 min prior to the addition of radioactive amino acids. L-[^35]S]methionine was used at a concentration of 50 µCi/ml.

For pulse-chase experiments, minicells were pulsed with L-[^35]S]methionine for 2 min at 37°C. Three volumes of chase mixture, consisting of 1% nonradioactive methionine in the labeling medium, was added, and the reaction was continued for various periods of time. At each time point, the reaction was stopped by the addition of an equal volume (four times the original) of a stop solution comprising 0.4 mg of chloramphenicol per ml, 0.4 M sodium azide, and 0.02 M 2,4-dinitrophenol. The samples were frozen immediately in a dry ice-acetone bath. Samples were processed by centrifugation to remove the inhibitors and excess label; the minicell pellet was processed either by radioimmunoprecipitation or by SDS-PAGE and autoradiography.

Radioimmunoprecipitations. For radioimmunoprecipitation analysis of products of the minicell analysis, the minicell pellet was suspended in 200 µl of 20 mM HEPES (pH 7.4)–1% SDS. The suspension was boiled for 5 min and then was subjected to centrifugation at 13,000 × g for 5 min to remove insoluble debris. The supernatant was transferred to another tube, and 20 mM HEPES (pH 7.4) was added to a total volume of 1 ml. Fifteen micrograms of a purified MAb or 10 µl of serum was added to this, followed by incubation (with rotation) overnight at 4°C. A 7.5-µg quantity of rabbit anti-mouse IgG was added to the tubes that contained mouse MAb; the mixture was incubated for 1 h at 4°C with agitation. A 200-µl sample of cell walls from Staphylococcus aureus Cowan 1 (18, 43) was added and incubated for 90 min at 4°C with mixing. The immune complexes were processed for SDS-PAGE (26, 28, 30). Sodium salicylate was used for fluorography (2).

SDS-PAGE and Western blotting (immunoblotting). SDS-PAGE was performed using a discontinuous gel system described by Marchitto et al. (22). Samples were boiled for 5 min in sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol prior to electrophoresis through a 4% stacking and a 12.5% separating gel. The gels were either stained with Coomassie brilliant blue or subjected to electrophoretic transfer to nitrocellulose (22). The nitrocellulose filters were incubated with either 10 µg of purified murine MAbs directed against T. pallidum antigens or with a 1:200 dilution of rabbit antiserum raised against the T. pallidum Triton X-114 detergent-phase proteins (31, 33). The appropriate horseradish peroxidase-conjugated secondary and tertiary antibody probes were each used at a 1:1,000 dilution. The chromogenic substrate was 4-chloro-1-naphthol (22).

Computer analysis. The Beckman Microgenie software version 5.0 (Beckman Instruments, Inc., Palo Alto, Calif.) (30) was used in conjunction with Version 5 of the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin-Madison) to overlay DNA sequences, as well as to analyze hydrophobicity and secondary structure.

RESULTS

Sequence of the 34-kDa antigen gene. A revised version of a preliminary map (43) of the pMN20 insert is shown in Fig. 1. The orientation is the same as that published previously (43). The sizes of the restriction fragments were deduced from the DNA sequence of the insert. The order of restriction sites is virtually identical to that on the previously determined map; with one correction. An ApaI site does not lie between the EcoRV site and the NsiI site as reported previously (43), but instead it lies nearly 40 base pairs (bp) upstream of (to the left of) the NsiI cleavage site. The 1,100-bp EcoRV fragment and the 910-bp AvaI fragment estimated before (43) are actually 1,157 and 900 bp long, respectively. The restriction map of the 34-kDa antigen gene is similar to that of TpD (16), indicating that the designations TpD and 34-kDa antigen refer to the same molecule (26). The plasmid pMN20 insert contains an open reading frame with the capacity to encode a polypeptide chain of 204 amino acids with a predicted molecular mass of 22,087 daltons (Fig. 2). The open reading frame begins at nucleotide 341 with an A.
methionine start codon and ends after nucleotide 952 with a UAG stop codon. Potential transcription and translation signals exist 5' to the predicted methionine initiation codon at nucleotide 341. A potential Pribnow (−10) box (15) exists from nucleotides 258 to 264, with a potential −35 region between nucleotides 337 and 242 (15). A potential ribosome-binding site (40) is found from −11 to −7 relative to the predicted initiation codon. The G+C content of the open reading frame is 55.2%.

The first 19 amino acids of the open reading frame constitute a potential leader peptide. The sequence Phe-Ser-Ala-Cys is similar to the consensus signal peptidase II recognition site, Leu-X-Y-Cys, which is characteristic of bacterial lipoproteins (47). The entire signal sequence has a predicted alpha-helical conformation (5) and is followed by a turn. The signal sequence has a composite +2 charge. Removal by processing of this putative leader peptide from the translation product yields a predicted mature protein of 158 amino acids with a molecular mass of 20,012 daltons. At a recent international meeting in Birmingham, England (The Biology and Pathogenicity of Treponemes, 11 April 1989), Leo Schouls and Jan van Embden reported the DNA sequence for the TpD gene. The conclusions here are identical to theirs.

**Amino acid sequencing of cyanogen bromide fragments of the 34-kDa antigen.** To confirm the correct reading frame, N-terminal amino acid microsequencing was performed on fragments isolated after cyanogen bromide cleavage of the 34-kDa molecule. Preliminary evidence indicated that the Triton X-114 detergent-phase proteins are blocked to Edman degradation. Because it was not possible to obtain purified 34-kDa antigen in amounts sufficient to isolate individual fragments for sequence determination, a mixed sequence was obtained from the cyanogen bromide-cleaved 34-kDa antigen (Table 1). The amino acids identified in each cycle of the Edman degradation were consistent with the predictions based on the deduced amino acid sequence. The consistency was especially apparent in the first five cycles of N-terminal sequencing (Table 1).

**Amino acid composition.** The predicted mature protein is 158 amino acids long. The protein is not markedly hydrophobic; only 28.1% of the residues are hydrophobic. The number of acidic residues (17.8%) is almost three times greater than the number of amino acids with basic side chains (6.5%). There are two cysteines in the deduced amino acid sequence. The calculated compositional PI of the mature 34-kDa antigen is 5.79; the observed isoelectric point is below 5.0 (26, 31), consistent with the abundance of acidic amino acids.

**Hydropathy and secondary structure analysis.** The entire deduced sequence was analyzed for secondary structure and hydrophobicity. The results of hydropathy analysis (19) are shown in Fig. 3. According to this analysis, the most obvious potential transmembrane domain is the signal sequence. The remainder of the molecule appears to be hydrophilic.

The peak of hydrophilicity that has its center near residue 83 is both the most likely epitope and has the highest probability of being on the surface of the protein (10, 30).

**Both the native and recombinant DNA-derived 34-kDa antigens partition into the detergent phase after extraction of intact cells with Triton X-114.** Treatment of intact *T. pallidum* with Triton X-114 followed by phase separation extracts a set of highly immunogenic membrane proteins into the detergent phase (7, 31, 33). The 34-kDa antigen of *T. pallidum* partitioned into the detergent phase after extraction in 2% Triton X-114, as assayed by Western blotting with MAb 3G9 (data not shown). Treatment of intact *E. coli* RR1(pMN20) with 2% Triton X-114, followed by phase separation, also extracted the 34-kDa antigen into the detergent phase; no 34-kDa antigen was detected in the aqueous phase after extraction of the recombinant host (data not shown).

**The 34-kDa protein localizes in both the inner and outer membranes of *E. coli*.** The 34-kDa antigen cofractionated with the major outer membrane proteins OmpF/C and OmpA into the cell envelope (data not shown). Treatment of the isolated *E. coli* cell envelope with 2% sarcosyl preferentially

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**TABLE 1. Sequence of mixed cyanogen bromide cleavage fragments of the 34-kDa antigen**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A, H, F, N, M*</td>
<td>A, H, F, N, M*</td>
</tr>
<tr>
<td>2</td>
<td>A, P</td>
<td>A, P</td>
</tr>
<tr>
<td>3</td>
<td>V, A, P, G</td>
<td>V, A, P, G</td>
</tr>
<tr>
<td>4</td>
<td>P, D, V</td>
<td>P, D, V'</td>
</tr>
<tr>
<td>5</td>
<td>A, G, P</td>
<td>A, G, P'</td>
</tr>
<tr>
<td>6</td>
<td>D, A, P</td>
<td>D', A', P</td>
</tr>
<tr>
<td>7</td>
<td>A, Q, H, D</td>
<td>A', Q, H, D</td>
</tr>
<tr>
<td>8</td>
<td>E, P, Y, A</td>
<td>E', P', Y, A'</td>
</tr>
<tr>
<td>9</td>
<td>G, S, E</td>
<td>G, S, E'</td>
</tr>
<tr>
<td>10</td>
<td>A, K, G</td>
<td>K', G</td>
</tr>
</tbody>
</table>

*The 34-kDa antigen was purified from *T. pallidum*.*

*Cycle of sequential Edman degradation.*

*The amino acids are those predicted for cyanogen bromide cleavage of the mature protein as deduced from the DNA sequence. The mature protein has five methionine residues. The methionine codons begin at nucleotides 434, 437, 557, 734, and 746 of the DNA sequence in Fig. 2. Amino acids are indicated by the standard one-letter code.*

*Results of sequential Edman degradation.*

*The presence of methionine in the first cycle of Edman degradation is the result of incomplete cyanogen bromide cleavage.*

*Uncertain but probable.*

---

![FIG. 1. Revised partial restriction enzyme map of the pMN20 insert encoding the *T. pallidum* 34-kDa immunogen. The insert was cloned into the PstI site of pBR322. The EcoRI site of pBR322 lies to the right of the figure. The leftmost PstI site lies internal to the GC tail at the cloning junction. The sizes (in base pairs) of the restriction enzyme fragments were derived from the DNA sequence. The thick arrow denotes the location, size, and direction of transcription and translation of the open reading frame.](http://iai.asm.org/)
FIG. 2. DNA and deduced amino acid sequences for the 34-kDa antigen. The designations −35, −10, and S/D denote sequences that correspond to putative promoter (−35 and −10) and ribosome-binding (S/D) sites. The GenBank accession number for this sequence is M27494.

FIG. 3. Hydrophathy analysis of the 34-kDa antigen by the method of Kyte and Doolittle (19). Analysis of the entire antigen, including the signal sequence, is shown. Positive values denote regions of relative hydrophlicity. The hydrophobic domain corresponds to the putative leader peptide. The averaging window is seven.

solubilized inner membrane proteins, leaving outer membrane proteins, especially OmpF/C and OmpA (13), in an insoluble fraction (Fig. 4A, lanes 3 and 4). Extraction of cell envelopes of E. coli RR1(pMN20) with 20% sarcosyl released a large proportion of the 34-kDa antigen into the soluble inner membrane fraction (Fig. 4B, lane 3). However, an approximately equal amount of 34-kDa antigen remained with the sarcosyl-insoluble outer membrane-enriched pellet (Fig. 4B, lane 4). Treatment of cell envelopes with 2% Triton X-100 in the presence of 10 mM MgCl₂ (39) also differentially solubilized inner and outer membrane proteins (Fig. 4A, lanes 5 and 6). Treatment of E. coli RR1(pMN20) cell envelopes with Triton X-100 plus 10 mM MgCl₂ released the 34-kDa antigen into the Triton X-100-soluble fraction; an equivalent amount remained in the insoluble fraction (Fig. 4B, lanes 5 and 6). In comparison, the E. coli OmpF/C and OmpA proteins remained in the insoluble pellet. If the envelopes of E. coli RR1(pMN20) are extracted with 2% Triton X-100 in the presence of 10 mM EDTA, both OmpF/C and OmpA becomes partially soluble (Fig. 4A, lanes 7 and 8) (38). The presence of EDTA increased the efficiency of solubilization of the 34-kDa antigen (Fig. 4B, lane 7), although a significant quantity remained in the detergent-insoluble pellet (Fig. 4B, lane 8) along with OmpF/C and OmpA. The results of treatment of E. coli RR1(pMN20) cell membranes with Triton X-114 were equivalent to those of extraction with Triton X-100 (data not shown). Furthermore, when membranes were extracted with 2% Triton X-114 and 10 mM EDTA, the solubilized OmpF/C and OmpA partitioned with the 34-kDa antigen into the detergent phase, as one would expect for integral membrane proteins (data not shown).

In the immunoblot of the 34-kDa antigen in E. coli cell envelope fractions (Fig. 4B), the antigen appears to have two forms. This is an artifact caused by the comigration of OmpA and the 34-kDa antigen. The abundance of OmpA partially distorted the electrophoretic migration of the 34-kDa antigen, excluding the latter from that region of the immunoblot. This particular region of negative stain was not observed with cytoplasmic membrane fractions in which no visible OmpA was present (compare Fig. 4B, lanes 5 and 6), supporting the contention that the negatively stained entity is OmpA.

The diffuse electrophoretic mobility of the 34-kDa antigen is apparent in Fig. 4 and has been described previously (16,
FIG. 4. SDS-PAGE and immunoblot analysis after detergent extraction of cell envelopes from recombinant E. coli. (A) SDS-PAGE gel stained with Coomassie brilliant blue. Lanes: 1, molecular size standards; 2, total cell membranes; 3, fraction solubilized by 2% sarcosyl; 4, sarcosyl-insoluble fraction; 5, fraction solubilized by 2% Triton X-100–10 mM MgCl₂; 6, Triton X-100–10 mM MgCl₂-insoluble fraction; 7, fraction solubilized by 2% Triton X-100–10 mM trisodium EDTA; 8, Triton X-100–10 mM EDTA-insoluble fraction. (B) Immunoblot of a duplicate gel with MAb 3G9. Lanes 1 through 8 are identical to those in panel A. Lanes 2 contained approximately 20 μg of membrane protein. The fractions from the detergent extractions were adjusted to the original extraction volumes, and equal volumes were loaded. The total protein content of each extraction pair was approximately 20 μg. The molecular size standards are phosphorylase b (92.5 kDa), bovine albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa). The band denoted OmpF is probably a mixture of OmpF and OmpC (11). E. coli OmpA (B) comigrated with the 34-kDa antigen, causing distortion of the electrophoretic mobility of the 34-kDa antigen and excluding stain from the relevant portion of the Western blot.

The 34-kDa molecule is processed in E. coli minicells. The results of a pulse-chase processing experiment are consistent with the existence of a cleaved signal peptide (Fig. 6). The 34-kDa antigen was synthesized as a higher-molecular-mass precursor of 37-kDa (Fig. 6) identical in migration to the in vitro-synthesized product (Fig. 5; data not shown). The proportion of the mature form of the 34-kDa protein increased as the time of chase increased. A fully mature form appeared after 60 min of chase. The maturation of the 34-kDa protein appeared to be a two-step process; the disappearance of the precursor correlated with the appearance of a discrete band 2 or 3 kDa smaller. This discrete band of approximately 34-kDa changed its migration such that it migrated as a diffuse smear from approximately 28 to 34 kDa, similar to the appearance of the mature antigen. Parallel experiments with minicells prepared from E. coli ORN103 containing pBR322 demonstrated that β-lactamase was processed more efficiently than the 34-kDa antigen and that no pBR322-derived product was immunoprecipitatable with the specific anti-34-kDa antigen MAbS (data not shown).

DISCUSSION

The data presented demonstrate that the 34-kDa antigen is a hydrophobic membrane protein of T. pallidum. Recently, Radolf et al. (31) demonstrated that treatment of intact organisms with the nonionic detergent Triton X-114 allows the identification of several hydrophobic, highly immunogenic proteins that constitute a major fraction of the treponemal membrane proteins. The 34-kDa antigen was one of the proteins in the detergent-rich hydrophobic phase (31).
The current results confirm and extend the previous observations. The 34-kDa antigen of *T. pallidum* cofractionates with the 47-kDa antigen (7, 31, 33) in Triton X-114 and is, therefore, one of the immunogenic integral membrane proteins of *T. pallidum*.

Several recombinant treponemal antigens localize into membrane compartments of *E. coli*. Fehninger et al. (11) described a recombinant 38-kDa antigen of *T. pallidum* that cofractionates with *E. coli* outer membrane proteins in sucrose gradients. The recombinant 47-kDa major membrane immunogen of *T. pallidum* localizes principally into the *E. coli* inner membrane (3). The 47-kDa antigen does become incorporated into the *E. coli* outer membrane when the gene is placed under the control of an inducible promoter (3). The 34-kDa antigen fractionated into the cell envelope of *E. coli*, indicating that it has the molecular signals to direct it into a membranous cellular compartment. Detergent treatment of purified *E. coli* membranes under conditions that are known to preferentially extract the inner membrane (13, 39) partially solubilized the 34-kDa antigen, suggesting that a substantial proportion of the antigen is located in the *E. coli* cytoplasmic membrane. The observation that extraction of *E. coli* cell envelopes under conditions that destabilize the outer membrane (38) was not sufficient to completely solubilize the 34-kDa antigen is consistent with the hypothesis that some of this antigen was present in the *E. coli* outer membrane. The combined results indicate that the 34-kDa antigen is a component of both cellular membranes of recombinant *E. coli*. Furthermore, the 34-kDa antigen partitioned into the detergent phase following extraction of *E. coli* clones with Triton X-114, indicating that the native and recombinant forms have similar hydrophobic properties. The *E. coli* major outer membrane proteins OmpF/C and OmpA, which can be solubilized partially by 2% Triton X-114 in the presence of EDTA, also partitioned with the 34-kDa antigen into the detergent phase, consistent with the contention that Triton X-114 phase separation discriminates between soluble and membrane proteins (7, 31). This result also strengthens the conclusion that the 34-kDa antigen is an integral membrane protein in both *T. pallidum* and *E. coli*.

Hydropathy analysis of the 34-kDa antigen revealed only one potential membrane-spanning domain. This region, the signal sequence of the 34-kDa antigen, is presumably absent from the mature peptide. Analysis of the remainder of the molecule revealed no other areas of obvious membrane-spanning potential. The sequences of BMP (8), TmpA (14), and the 47-kDa antigen (17) also were analyzed for hydrophobicity. Except for the signal peptides in BMP and TmpA, there are no obvious transmembrane regions in any of the previously published sequences. There is abundant evidence, however, that both the 34- and 47-kDa antigens are very hydrophobic proteins. The absence of unequivocal transmembrane regions in both the 34- and 47-kDa antigens does not necessarily indicate that the proteins are hydrophilic; it may simply indicate that the protein structure computer programs lack sufficient predictive power (36). The covalent attachment of lipid (47) also could provide an alternative means of conferring hydrophobicity to the 34-kDa antigen, as well as to other immunogens of the membrane protein-enriched Triton X-114 phase. Recent evidence suggests that the immunogenic membrane proteins of *T. pallidum* found within the Triton X-114 detergent phase are proteolipids (2). Furthermore, both *E. coli* and *T. pallidum* incorporate exogenous [3H]palmitic acid into the 34-kDa antigen (M. A. Swancutt, J. D. Radolf, and M. V. Norgard, submitted for publication).

Analysis of the deduced amino acid sequence of the 34-kDa antigen predicts the existence of a leader peptide (45). There is evidence that the signal peptide is used. Pulse-chase experiments with *E. coli* minicells demonstrated that the primary product is processed to a lower-molecular-mass form. The processed form appears first as a discrete product of approximately 34 kDa, and it subsequently migrates in the mature form over a range of molecular masses. The rate of maturation of the 34-kDa protein appears to be 

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**FIG. 5.** Comparison of the *T. pallidum* native 34- and 47-kDa antigens with the primary translation products produced by *E. coli* cell-free transcription-translation. Reaction products of the cell-free transcription-translation system were mixed with *T. pallidum*, coelectrophoresed in SDS-PAGE, and transferred to nitrocellulose filters. The filter was first subjected to autoradiography and then probed with MAbs 11E3 and 3G9 to detect the 47- and 34-kDa antigens, respectively. Lanes 1 through 4 show the results of autoradiography: lane 1, the same four radioactive high-molecular-mass standards as in Fig. 4 plus cytochrome c (12.3 kDa); lane 2, pBR322 (negative control); lane 3, pMN20 (expressing the 34-kDa antigen); lane 4, pNC81 (encoding the 47-kDa antigen). Lane 5, Immunoblot analysis of the contents of lane 4. The asterisk is located next to the 37-kDa primary translation product encoded by pMN20 (lane 3).

**FIG. 6.** Time-dependent processing of the 34-kDa antigen by *E. coli* minicells. Minicells containing pMN20 were pulse-labeled with [35S]methionine and chased by the addition of nonradioactive methionine for the time (minutes) indicated (numbers above each lane). After the reactions were stopped, the reaction products were immunoprecipitated by MAb 5C11. The numbers 37 and 34 at the left denote the primary and mature forms of the antigen, respectively, in kilodaltons.
slow relative to that of the pBR322-encoded β-lactamase. This is consistent with previous observations that the treponemal basic membrane protein is inefficiently processed in E. coli maxicells (8).TmpA also appears to mature slowly (14). The molecular reasons for this are unclear but probably reflect differences in the treponemal and enterobacterial export machinery. The sequence Phe-Ser-Ala-Cys at positions 17 through 20 of the deduced protein sequence is similar to the consensus sequence Leu-X-Y-Cys, which is found in bacterial lipoproteins (47). This sequence probably acts as the target sequence for the covalent addition of a diacylglycerol to the cysteine of bacterial lipoproteins and for the subsequent processing by signal peptidase II. Although phenylalanine is unusual in the -3 position, the assignment is probably correct because globomycin, a specific signal peptidase II inhibitor (47), inhibits the maturation of the 34-kDa antigen in both E. coli and T. pallidum (Swancutt et al., submitted). It is interesting that the published sequence of TmpA also contains a consensus signal peptidase II cleavage site (14), suggesting that TmpA also may be a lipoprotein.

It is evident that the 34-kDa antigen has a unique electrophoretic profile, migrating over a 6-kDa range of apparent molecular masses. This profile is unique (4, 24, 44), and the electrophoretic behavior accounts for the failure to readily identify this protein in Coomassie blue-stained gel profiles of whole treponemes. The comigration of other polypeptides, especially endoflagellar proteins, in the same area of one-dimensional polyacrylamide gels also obscures detection of the 34-kDa antigen in immunoblots using either human or rabbit syphilitic sera (26). Furthermore, several discrete proteins in the T. pallidum Triton X-114 detergent phase also comigrate with the 34-kDa antigen (7, 31). The recombinant 34-kDa antigen showed identical electrophoretic behavior. The heterogeneous electrophoretic behavior of the recombinant 34-kDa antigen also accounts for the failure to identify a species corresponding to the 34-kDa protein in Coomassie blue-stained profiles of either cell envelopes or membrane fractions from recombinant E. coli clones. Hindersson et al. (16) have noted that this molecule does not stain well with Coomassie brilliant blue and have postulated that poor staining is an explanation for the failure to easily detect this antigen. The similar electrophoretic profiles of both the recombinant and native 34-kDa proteins suggest that this behavior is a function of the final structure of the protein.

The molecular basis for the unique electrophoretic behavior of the 34-kDa antigen in SDS-PAGE is unknown. The heterogeneous (smearsed) electrophoretic profile could be a consequence of the primary amino acid sequence of the 34-kDa antigen. This explanation is unlikely because the primary translation products from both the cell-free translation system and from the E. coli-derived minicells migrated as a distinct 37-kDa species. The heterogeneous profile is thus a characteristic of the mature protein. Alternatively, proteolytic cleavage during maturation could generate a diverse population of molecules which migrate heterogeneously because of variability in molecular masses. However, one would also expect proteolysis to generate a family of peptides with differing isoelectric points. The observation that the 34-kDa antigen behaves homogeneously in the isoelectric focusing portion of a two-dimensional gel system (26, 31) suggests that proteolysis is probably not the reason the 34-kDa antigen migrates over a range of molecular masses. Furthermore, inclusion of protease inhibitors in T. pallidum extraction mixtures has no effect on the electrophoretic mobility of the 34-kDa antigen (M. A. Swancutt, unpublished results), suggesting that proteolysis during extraction is not responsible for the heterogeneous electrophoretic mobility. The covalent addition of fatty acid to the peptide may be responsible for the heterogeneous electrophoretic mobility of the mature 34-kDa antigen. It seems unlikely, however, that the addition of lipid can be solely responsible for the aberrant electrophoretic profile of the 34-kDa antigen, especially when one considers that such well-characterized E. coli lipoproteins as the peptidoglycan-associated lipoprotein (4), the cytoplasmic membrane 28-kDa lipoprotein (48), and the murein lipidprotein (47), as well as the other T. pallidum detergent-phase lipoproteins (2), migrate as discrete entities in one-dimensional SDS-PAGE. Another explanation is that the final tertiary structure of the mature molecule is not completely denatured by SDS, with the result that some molecules migrate more rapidly in SDS-PAGE due to a more compact three-dimensional structure. Preliminary experiments support the latter possibility; incorporation of 4 M urea as a chaotropic agent into the SDS-PAGE gel system retards the mobility of the 34-kDa antigen so that it migrates as a more discrete band rather than as a diffuse smear (M. A. Swancutt, unpublished results). Further experiments are needed to elucidate the origin of the characteristic mobility of the 34-kDa antigen.

There is a discrepancy of approximately 15 kDa between the apparent mobility of the 34-kDa antigen and its deduced molecular mass. Although the difference between observed and predicted molecular masses seems abnormally large, a discrepancy of this size has been reported for the E. coli lactose permease, a conventional membrane protein (9). The H.8 lipoprotein antigen of Neisseria gonorrhoeae (1) also shows a smeared electrophoretic profile in SDS-PAGE; interestingly, the calculated molecular mass of the 76-residue H.8 neisserial lipoprotein is approximately 6.9 kDa, compared with the observed apparent molecular mass of 20 kDa (1). Because the covalent addition of lipid does not cause molecular size aberrancies of this magnitude in other bacterial lipoproteins (4, 47, 48), lipid is probably not responsible for the greatly retarded mobility of either the H.8 protein or the 34-kDa antigen in conventional SDS-PAGE systems. Furthermore, the in vitro-derived 37-kDa primary product is not modified by the addition of lipid (Swancutt et al., submitted) and also shows a size discrepancy of the same magnitude. The high apparent molecular mass of the 34-kDa antigen is probably a result of the amino acid composition of the protein. The 34-kDa antigen is an acidic protein due to the preponderance of aspartate and glutamate residues. Neither of these acidic amino acids binds SDS effectively (20). In this context, it is relevant that none of the amino acids of the H.8 pentapeptide repeat, Pro-Ala-Ala-Glu-Ala (1), has significant binding affinities for SDS (20). A decrease in the amount of bound SDS could account for the decreased electrophoretic mobility of both the neisserial H.8 protein and the T. pallidum 34-kDa membrane protein. Furthermore, it is of interest that the replacement of a tyrosine residue by an aspartate causes the E. coli maltose-binding protein to migrate with a molecular mass approximately 1 kDa greater than that of the wild-type protein (6). Therefore, diminished binding of SDS by the 34-kDa antigen can account for both the aberrant observed molecular mass and the heterogeneous electrophoretic profile. We have retained the 34-kDa nomenclature to be consistent with the previous literature (26).

Earlier evidence indicated that the 34-kDa antigen was expressed from recombinant plasmids independent of the orientation of the insert DNA with respect to the vector (43).
Analysis of the DNA sequence of the region upstream of the 34-kDa antigen gene revealed the existence of sequences that probably function as transcription and translation signals. Sequences that correspond to the −35 and −10 E. coli promoter sequences (5), as well as to a potential E. coli ribosome-binding site (40), probably account for expression of the gene product in E. coli; the conservation may indicate that T. pallidum uses similar sequences for gene expression. The 34-kDa antigen is an integral membrane protein that displays apparently identical biochemical properties in a manner that is independent of the host organism. The DNA sequence of the 34-kDa antigen gene provides insight into the processing and maturation of the protein, as well as hypotheses to explain the hydrophobic characteristics of the molecule. Knowledge of the amino acid sequence also allows the design of rational experiments to identify the immunodominant epitopes that are the basis for the immunogenicity of the molecule. The data presented here demonstrate the validity of using recombinant DNA approaches, in conjunction with direct investigations of T. pallidum, to elucidate the macromolecular structure of the T. pallidum cell envelope and its relationship to the pathogenesis and immunology of syphilis.

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