INFECTION AND IMMUNITY, Apr. 1993, p. 1202–1210
0019-9567/93/041202-09$02.00/0

Vol. 61, No. 4

# Lipid Modification of the 17-Kilodalton Membrane Immunogen of *Treponema pallidum* Determines Macrophage Activation as well as Amphiphilicity

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Received 24 September 1992/Accepted 10 January 1993

**A murine monoclonal antibody specific for a 17-kDa major membrane immunogen of *Treponema pallidum* was used to select recombinant *Escherichia coli* clones expressing the molecule from a *T. pallidum* genomic library. Sequence analysis of the structural gene for the immunogen (designated *tpp17*) revealed a 468-bp open reading frame encoding a polypeptide of 156 amino acids with a calculated molecular mass of 16,441 Da. The deduced amino acid sequence included a putative leader peptide terminated by a consensus tetrapeptide for the modification and processing of prokaryotic lipoproteins. Immunoprecipitation of the cloned immunogen radiolabeled with [$^3$H]palmitate confirmed that it was a lipoprotein. The amino acid sequence also predicted that the mature protein contains four cysteine residues in addition to the lipid-modified cysteine of the N terminus. The existence of disulfide-bonded multimeric forms of the native immunogen was demonstrated by immunoblotting *T. pallidum* solubilized in the presence and absence of 2-mercaptoethanol. Triton X-114 phase partitioning of a nonlipidated form of the 17-kDa immunogen cleaved from a glutathione *S*-transferase fusion protein demonstrated that lipid modification is responsible for the immunogen's hydrophobic character. The same nonlipidated form of the immunogen also was used to demonstrate that lipid modification is essential for the molecule's ability to stimulate production of tumor necrosis factor alpha by murine macrophages. We conclude that covalently attached fatty acids not only anchor *T. pallidum* lipoproteins to spirochetal membranes but also confer upon these molecules the ability to activate immune effector cells.**

The parasitic strategies that enable *Treponema pallidum* subsp. *pallidum*, the etiologic agent of venereal syphilis, to evade host defenses are poorly understood. It is well recognized that the bacterium provokes an intense cellular and humoral immune response during syphilitic infection and that the resulting inflammation is responsible for many if not all of the disease's clinical manifestations (29, 52). At the same time, this immune response appears to have a limited ability to prevent dissemination and persistence of the spirochete. Our strategy for elucidating this paradoxical host-parasite relationship is analysis of *T. pallidum* ultrastructure (12, 41) in parallel with molecular and immunological characterization of important treponemal immunogens.

We and others previously reported on the use of phase partitioning with the nonionic detergent Triton X-114 as a convenient method for isolating the major membrane immunogens of *T. pallidum* (13, 38, 39). Subsequently, it was demonstrated that these highly immunogenic membrane proteins are covalently modified with lipids (i.e., they are lipoproteins) (9, 24, 36, 45, 48). Among these lipoproteins was a 17-kDa molecule identified initially by its intense reactivity with human syphilitic sera (3, 4, 19, 22) and later demonstrated to elicit a strong proliferative response in splenocytes from rabbits with experimental syphilis (2). Most recently, we used a murine macrophage-like cell line permanently transfected with a construct consisting of a tumor necrosis factor alpha (TNF-α) promoter upstream of a chloramphenicol acetyltransferase (CAT) reporter gene (7) to demonstrate that this lipoprotein can stimulate macrophages to produce TNF-α (40), a seminal mediator of inflammatory processes (8). Taken together, these data indicate that this molecule plays a potentially important role in syphilis pathogenesis. In keeping with the investigative strategy outlined above, we cloned the 17-kDa immunogen in *Escherichia coli* and used the data obtained by nucleotide analysis to examine the effects of lipid modification on its physical and immunological properties.

## 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 (38). *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. DH5αF′ (Gibco/BRL) was used for sequence analysis of M13 subclones. Plasmids pUC18 (53) and pGEX-2T (46) (Pharmacia LKB Biotechnology, Piscataway, N.J.) were used for subcloning experiments, deletion analysis, and protein fusion experiments.

**Extraction and phase partitioning with Triton X-114.** Extraction and phase partitioning with Triton X-114 have been described previously (35, 38). Briefly, $1 \times 10^9$ freshly harvested *T. pallidum* cells, $5 \times 10^8$ freeze-thawed *E. coli* DH5α transformants, or 20 μg of purified nonlipidated 17-kDa immunogen was added to a 2% solution of Triton X-114 in phosphate-buffered saline (PBS; pH 7.4). Bacterial cells were extracted overnight at 4°C, and insoluble debris was

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then removed by centrifugation at 50,000 × g for 30 min. The supernatants were collected and phase separated. The respective phases were collected, washed five times, and then precipitated with 10 volumes of ice-cold acetone. The *T. pallidum* detergent-phase proteins obtained in this manner are referred to here as DPPs.

**Immunological reagents.** Murine monoclonal antibody (MAb) 19G3 directed against the 17-kDa protein was generated as follows. A female BALB/c mouse was injected intraperitoneally for six consecutive months with $10^7$ *T. pallidum* in Ribi adjuvant. This was followed 2 months later by intraperitoneal and intravenous immunization with a total of $10^7$ organisms in PBS. Two months later, the mouse was injected intravenously and intraperitoneally with 5 μg of purified native 17-kDa immunogen in PBS. The mouse was sacrificed 3 days later, and its spleen was removed for the production of hybridomas. Hybridomas producing antibodies reactive with *T. pallidum* were identified by radioimmunoassay as previously described (43), and their molecular specificities were determined by immunoblot analysis.

Samples of human syphilitic sera were obtained from patients with classic manifestations of secondary syphilis and reactive treponemal and nontreponemal serodiagnostic tests. Horseradish peroxidase conjugated to rabbit anti-mouse, goat anti-rabbit, and rabbit anti-goat immunoglobulin G and protein A were purchased from Zymed Laboratories, Inc. (San Francisco, Calif.). To generate polyclonal antisera directed against purified glutathione *S*-transferase (GST) and GST fusion proteins, two rabbits each were primed with 100 μg of purified protein in 1 ml of PBS mixed 1:1 with complete Freund's adjuvant (total volume, 2 ml) at two intramuscular and two subcutaneous sites. The animals were boosted twice at the same sites during 2-week intervals with 50 μg of antigen in 1 ml of PBS mixed 1:1 with Freund's incomplete adjuvant and were exsanguinated 2 weeks after the second boost. The specificities of each antiserum were confirmed by immunoblot analysis with *T. pallidum* and *E. coli* whole-cell lysates and purified GST. The polyclonal antisera were absorbed extensively with sonicates of *E. coli* DH5α whole cells to remove background immunoreactivity.

**Identification of recombinant clone expressing 17-kDa immunogen.** A *T. pallidum* genomic library constructed in pBR322 (31) was screened initially with pooled human syphilitic sera by radioimmunocolony blot assay (RICB). Recombinant clones identified in this manner then were rescreened with MAb 19G3 by the RICB technique.

**Generation of nested deletions to localize gene encoding 17-kDa lipoprotein.** Nested deletions of pAE47 were generated by digesting the plasmid to completion with *Sac*I and *Bam*HI. This procedure was followed by exonuclease III digestion for various times and treatment with mung bean nuclease by using the Exo III/Mung Bean kit according to the manufacturer's (Stratagene, La Jolla, Calif.) instructions. The resulting plasmids were transformed into DH5α and screened by immunoblot analysis.

**DNA sequence analysis.** DNA sequence analysis of subclones in M13mp18 and M13mp19 (56) was performed by using the dideoxy termination method of Sanger et al. (44) with modified T7 DNA polymerase (Sequenase 2.0; United States Biochemicals Corp., Cleveland, Ohio). The following sequencing primers were used in addition to the standard M13 primers: 5′-CTCGTGCACAACCGTGTGTC-3′ (nucleotides 168 to 187), 5′-CGGCCCTCCTCTTCCCCTCG-3′ (complementary to nucleotides 637 to 656), 5′-GGAGTTAC TGTCTATCAGCAC-3′ (complementary to nucleotides 466 to 486), and 5′-CGTCACAGTCGTATCGATTCC-3′ (complementary to nucleotides 277 to 297). Nucleotide and amino acid sequence analyses were conducted with the University of Wisconsin Computer Genetics Group software package (15).

**Production of nonlipidated 15- and 17-kDa proteins as fusions with GST.** Constructs expressing the nonlipidated 15- and 17-kDa proteins were produced by cloning DNA fragments encoding the mature portions of the 15- and 17-kDa immunogens into the *Bam*HI and *Sma*I sites of pGEX-2T. These corresponded to nucleotides 384 to 755 (amino acids 18 through 141) of *tpp15* (36) and nucleotides 163 to 567 (amino acids 22 to 156) of *tpp17*. The respective fragments were produced by the polymerase chain reaction with the following synthetic oligonucleotide primers: 5′-TTGGATCC TGTTCATTTAGTTCTATC-3′ (*Bam*HI site plus nucleotides 384 to 401) and 5′-GTCTACCTGCTAATAATGGC-3′ (complementary to nucleotides 760 to 741) for *tpp15* and 5′-CGGGATCCTGTGTCTCGTGCACAACCGTGTGT-3′ (*Bam*HI site plus nucleotides 163 to 186) and 5′-CGGCCCT CCTCTTCCCCTCG-3′ (complementary to nucleotides 637 to 656) for *tpp17*. The resulting constructs were designated pLP440 and pDA500, respectively. The sequences through the fusion joints were verified with the sequencing primer 5′-CCTTTGCAGGGCTGGCAAGC-3′ (nucleotides 861 to 880 of pGEX-2T).

**Protein purification.** The native 15- and 17-kDa immunogens were purified from DPPs by chromatofocusing as previously described (35). Briefly, a column (1 by 10 cm) of PBE 94 (Pharmacia LKB Biotechnology) gel was prepared according to the manufacturer's instructions and overlaid with 1 cm of swollen Sephadex G-25 resin (Pharmacia). The gel was first equilibrated with 10 bed volumes of 0.025 M imidazole-HCl (pH 7.4) followed by the same volume of 0.025 M imidazole-HCl (pH 7.4) containing 1% Triton X-114. Approximately 500 μg of DPPs was diluted with an equal volume of 0.025 M imidazole-HCl (pH 7.4) and applied to the column. The proteins were eluted by using Polybuffer 74 HCl (pH 4.0; Pharmacia). Column fractions were collected and analyzed by immunoblotting.

GST and GST fusion proteins were purified by affinity chromatography on a glutathione-agarose matrix according to the manufacturer's instructions (46). To liberate the nonlipidated treponemal proteins, the fusion proteins were cleaved with thrombin before or after elution from the agarose affinity matrix (46). For macrophage activation experiments, purified native and nonlipidated immunogens were passed through a Pyrobind syringe tip (Sepracor Inc.) to remove any residual endotoxin from the protein preparations. Protein concentrations were determined by using the bicinchoninic acid protein assay micromethod (Pierce Chemical Co., Rockford, Ill.).

**SDS-PAGE and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (27). Samples were boiled for 5 min in final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with or without 5% (vol/vol) β-mercaptoethanol prior to electrophoresis through 2.4% stacking and 12.5% separating gels. Gels were then either stained in Coomassie brilliant blue or transferred electrophoretically to 0.2-μm-pore-size nitrocellulose for immunoblotting (51).

**[$^3$H]palmitate radiolabeling and immunoprecipitation of 17-kDa gene product.** Single colonies of *E. coli* DH5α (pUC18) and *E. coli* DH5α(pAE47) were each inoculated into 200-ml portions of minimal medium containing ampicillin (100 μg/ml) and thiamine (10 μg/ml) and grown to an

optical density at 600 nm of 0.10. [9,10(*n*)-$^3$H]palmitate (300 μCi; 60 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each culture and then incubated further with shaking to an optical density at 600 nm of 0.8. The cells then were harvested, washed twice in PBS, suspended in 2 ml of PBS, and lysed by two cycles of freeze-thawing. Triton X-114 was added to the cell lysates (final detergent concentration, 2% [vol/vol]) for phase partitioning as described above. Two 0.1-ml portions of each detergent phase were diluted with PBS to a volume of 1 ml, and then 50 μl of GammaBind G-agarose (Genex Corp., Gaithersburg, Md.) was added. The suspensions were rocked at 4°C for 2 h to clear material that would bind nonspecifically to the beads. After removal of the beads by centrifugation for 5 min at 500 × *g*, 50 μl of rabbit antiserum directed against either the 15- or the 17-kDa protein–GST fusion protein and 50 μl of GammaBind G-agarose were added to each detergent phase. The suspensions were rocked overnight at 4°C, and beads were harvested. The beads were washed three times in PBS and boiled for 5 min in final sample buffer for SDS-PAGE and fluorography.

*T. pallidum* was metabolically radiolabeled with [$^3$H]palmitate as described by Chamberlain et al. (9). After overnight incubation, cells were extracted extensively with 2% Triton X-114 and then phase partitioned. Portions of the diluted detergent phase were immunoprecipitated as described above.

**Protein sequencing.** To obtain sequence from the native 17-kDa immunogen, DPPs were separated on a 12.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.). The 17-kDa immunogen was visualized by staining with Ponceau S and then excised for N-terminal sequence analysis. Two different methods were used to obtain internal protein sequence after it was determined that the N terminus of the native immunogen was blocked to Edman degradation. To obtain mixed sequence, the protein on the polyvinylidene difluoride membrane was subjected to in situ digestion with cyanogen bromide (CNBr) followed by automated Edman degradation. Additional internal sequence was obtained by transfer of DPPs to 0.2-μm-pore-size nitrocellulose followed by solid-phase tryptic digestion. The eluted peptide fragments were separated by reverse-phase high-performance liquid chromatography (HPLC) on a model 130A Applied Biosystems chromatography with a Brownlee RP300 C8 column (2.1 by 100 mm). Separation was performed in 0.1% trifluoroacetic acid using a 0 to 50% acetonitrile gradient for 120 min with a flow rate of 50 μl/min. Peaks were collected onto 1-cm disks of Whatman GF/C paper and then sequenced by automated Edman degradation. N-terminal sequences of the nonlipidated 15- and 17-kDa molecules were obtained by automated Edman degradation following thrombin cleavage of the GST fusion protein, separation of the cleavage products by SDS–PAGE, and transfer to polyvinylidene difluoride paper.

**Induction of CAT in transfected RAW 264.7 cells.** RAW 264.7 murine macrophage-like cells permanently transfected with a CAT reporter gene controlled by the TNF-α promoter were passaged as described elsewhere (7, 40). *E. coli* lipopolysaccharide (LPS) (strain O127:B8) was obtained from Difco (Chicago, Ill.). To induce CAT, transfected cells were stimulated for 16 h with 1 μg of LPS, purified native lipoproteins, or purified recombinant nonlipidated proteins per ml in the presence or absence of 10 μg of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) per ml. CAT assays were performed on RAW 264.7 cell lysates as previously described by Gorman et al. (17). Following induction, cells were lysed by freeze-thawing in 120 μl of 0.25 M Tris-Cl (pH 7.5) and centrifuged for 5 min in a microcentrifuge. Supernatants were then collected, heated for 5 min at 65°C, and recentrifuged. A 60-μl portion of each cell supernatant, 37.5 × $10^{-3}$ μCi of [$^{14}$C]chloramphenicol (New England Nuclear; 50 μCi/mmol), and 20 μl of 4 mM acetyl coenzyme A were added to 100 μl of 0.25 M Tris-HCl (pH 7.5) and incubated at 37°C for 2 h. The reaction was then stopped by the addition of 1 ml of cold ethyl acetate, and the phases were separated. The organic phase was dried, suspended in 10 μl of ethyl acetate, and then subjected to chromatography on silica gel thin-layer plates with chloroform-methanol (95/5).

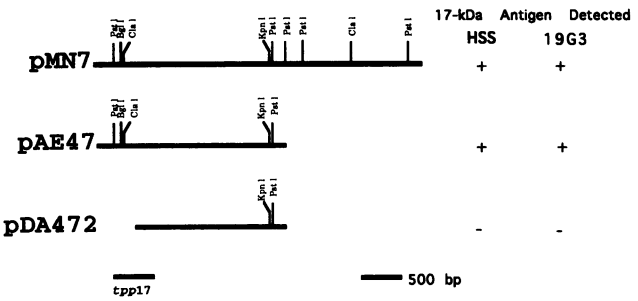


FIG. 1. Localization of the cloned gene encoding the 17-kDa lipoprotein immunogen of *T. pallidum* (*tpp17*). Shown are partial restriction maps of the parental plasmid pMN7 (pBR322 vector) and pUC18 derivatives along with immunoreactivities of the respective *E. coli* transformants with pooled human syphilitic sera (HSS) and MAb 19G3.

**LAL assay.** *Limulus* amebocyte lysate (LAL) assays of the purified native and nonlipidated 15- and 17-kDa proteins were performed by using the Sigma LAL kit and following the recommended protocol. This assay routinely detects a minimum of 20 pg of endotoxin per ml.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper has been submitted to the GenBank data base and assigned the accession number M74825.

## RESULTS

**Characterization of *E. coli* transformants expressing the 17-kDa lipoprotein immunogen.** A *T. pallidum* genomic library constructed in pBR322 was screened by RICB assay with pooled human syphilitic sera to identify clones expressing treponemal immunogens (31). These recombinants subsequently were rescreened with MAb 19G3, an immunoglobulin G2b specific for the 17-kDa lipoprotein of *T. pallidum*, to identify clones expressing this immunogen. One clone, designated pMN7 (49), reacted with MAb 19G3 by RICB assay and expressed a 17-kDa protein on Western blots (immunoblots; data not shown). The 3.7-kb insert of pMN7 was mapped (Fig. 1), and fragments were subcloned into pUC18 for rescreening with MAb 19G3. A subclone containing a 1.8-kb *Pst*I fragment (pAE47) was identified in this manner (Fig. 1). Immunoblot analysis with MAb 19G3 and pooled human syphilitic sera confirmed that pAE47 expressed a 17-kDa immunogen with an SDS-PAGE mobility identical to that of the native molecule along with a 19-kDa protein (presumably unprocessed precursor) (Fig. 2). Interestingly, larger forms of the immunogen also were identified in *T. pallidum* whole-cell lysates, *T. pallidum* DPPs, and *E. coli* DH5α(pAE47) whole-cell lysates, although the apparent

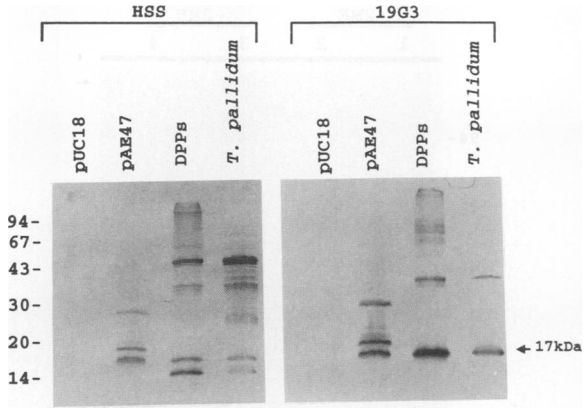


FIG. 2. Immunoblot analysis of the native and recombinant 17-kDa *T. pallidum* immunogen. *T. pallidum* whole cells, *E. coli* DH5α harboring plasmids pUC18 or pAE47, and *T. pallidum* membrane proteins isolated by Triton X-114 phase partitioning (DPPs) were immunoblotted with either pooled human syphilitic sera (HSS) or MAb 19G3. Molecular mass standards in kilodaltons are shown at left.

molecular masses of the larger *T. pallidum* bands differed from those of the recombinant *E. coli* clone (Fig. 2). Nested deletions were generated to localize the structural gene within the 1.8-kb insert of pAE47. A clone (pDA472) with a deletion of approximately 200 bp from the left *Pst*I cloning site of pAE47 was nonreactive with both MAb 19G3 and pooled human syphilitic sera (Fig. 1). The failure to detect expression of any antigen, including truncated forms, by pDA472 suggested that the deleted fragment included the promoter for the 17-kDa immunogen gene.

**Nucleotide sequence of the 17-kDa immunogen.** On the basis of the above-described deletion analysis, sequencing was initiated from the left *Pst*I cloning site of pAE47 (Fig. 1). An open reading frame of 468 bp was identified along with putative upstream *cis*-acting control elements within the first 650 bp (Fig. 3). The open reading frame of the 17-kDa lipoprotein structural gene, designated *tpp17*, begins with a methionine start codon at nucleotide 100 and ends with a UAG stop codon after nucleotide 567, thereby encoding a polypeptide of 156 amino acids with a predicted molecular mass of 16,441 Da (Fig. 3). The first 21 amino acids of the open reading frame encode a potential leader peptide ending with Ser-Ala-Leu-Cys, a sequence that conforms to the consensus required for the modification and processing of prokaryotic prolipoproteins (55). The predicted mature polypeptide consists of 135 amino acids with a molecular mass of 14,438 Da. It contains 64 (47.4%) hydrophobic, 16 (11.8%) acidic, and 21 (15.6%) basic residues with a calculated pI of 8.33. The deduced mature polypeptide also contains five cysteines. A complete search of the GenBank and EMBL data bases failed to identify any significant sequence homologies with *tpp17*. Hydrophobicity analysis, performed according to the algorithm of Kyte and Doolittle (26), failed to identify amino acid stretches, other than that of the leader peptide, likely to encode transmembrane domains (data not shown). Utilizing the parameters of Chou and Fasman (11), five potential B-cell determinants centered at amino acids 33, 64, 111, 138, and 142 were identified within the mature polypeptide. Three potential T-cell epitopes, identified by using the algorithm of Margalit et al. (30) (window size of 11), were located at amino acids 23 to 33, 119 to 129, and 128 to 138.

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1                                                                  50
TGCGCTCTTCTCTCACTTTTGGCAAGAGGGAGCGTAACTTTTGCATCGTTGGGCTTTTCTCCC
                                                  -35

                                          100
CTGAGTCTTACAGACGTGACAAGGGGGTTTTCTGTGATG AAA GGA TCT GTC CGC GCG
          -10          S.D.              MET LYS GLY SER VAL ARG ALA
                                          1
                                          150
CTG TGC GCG TTC CTT GGT GTT GGA GCG CTC GGT AGC GCT TTG TGT GTC
LEU CYS ALA PHE LEU GLY VAL GLY ALA LEU GLY SER ALA LEU CYS VAL
                                                    20
                                                  200
TCG TGC ACA ACC GTG TGT CCG CAC GCC GGG AAG GCC AAA GCG GAA AAG
SER CYS THR THR VAL CYS PRO HIS ALA GLY LYS ALA LYS ALA GLU LYS

                                                250
GTA GAG TGC GCG TTG AAG GGA GGT ATC TTT CGG GGT ACG CTA CCT GCG
VAL GLU CYS ALA LEU LYS GLY GLY ILE PHE ARG GLY THR LEU PRO ALA
 40
                                                300
GCC GAT TGC CCG GGA ATC GAT ACG ACT GTG ACG TTC AAC GCG GAT GGC
ALA ASP CYS PRO GLY ILE ASP THR THR VAL THR PHE ASN ALA ASP GLY
                60
                                                      350
ACT GCG CAA AAG GTA GAG CTT GCC CTT GAG AAG AAG TCG GCA CCT TCT
THR ALA GLN LYS VAL GLU LEU ALA LEU GLU LYS LYS SER ALA PRO SER
                                80
                                                          400
CCT CTT ACC TAT CGC GGT ACG TGG ATG GTA CGT GAA GAC GGA ATT GTC
PRO LEU THR TYR ARG GLY THR TRP MET VAL ARG GLU ASP GLY ILE VAL
                                                        100
                                                      450
GAA CTC TCG CTT GTG TCC TCG GAG CAA TCG AAG GCA CCG CAC GAG AAA
GLU LEU SER LEU VAL SER SER GLU GLN SER LYS ALA PRO HIS GLU LYS

                                                    500
GAG CTG TAC GAG CTG ATA GAC AGT AAC TCC GTT CGC TAC ATG GGC GCT
GLU LEU TYR GLU LEU ILE ASP SER ASN SER VAL ARG TYR MET GLY ALA
120
                                                          550
CCC GGC GCA GGA AAG CCT TCA AAG GAG ATG GCG CCG TTT TAC GTG CTC
PRO GLY ALA GLY LYS PRO SER LYS GLU MET ALA PRO PHE TYR VAL LEU
                140
                                                        600
AAA AAA ACA AAG AAA TAGTTAAGTCTGCCCCTTCCCTTCCGTTCCTTCGGGCTCTGTG
LYS LYS THR LYS LYS ***

                                    650
CGCCCCCTTCGGCAGTACCACGGTCACGAGGGGAAGAGGAGGGCCGACCT
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FIG. 3. DNA and amino acid sequences of the 17-kDa immunogen. Nucleotides are numbered above the sequence, and deduced amino acids are numbered below it. The putative promoter (−35 and −10), Shine-Dalgarno (S.D.) region, and lipoprotein consensus modification and processing sequence are indicated by boxes. Asterisks denote the termination codon. The proposed signal peptidase II processing site is denoted by an arrow between amino acids 21 (Leu) and 22 (Cys). Portions of the deduced amino acid sequence confirmed by protein sequence analysis are shown in boldface; italicized boldface indicates amino acid sequence obtained from the nonlipidated 17-kDa immunogen.

**Protein sequence analysis.** Protein sequence analysis was performed to confirm that the correct reading frame of the nucleotide sequence had been identified. As expected for a prokaryotic lipoprotein (55), the N terminus of the purified native molecule was blocked to Edman degradation. Internal sequence was obtained from a mixture of CNBr fragments as well as from individual tryptic fragments separated by HPLC. All of the sequences were in complete agreement with that predicted by nucleotide sequence analysis (Fig. 3). To obtain amino acid sequence closer to the N terminus of the molecule, the predicted mature portion of *tpp17* was fused downstream and in frame from the GST gene of pGEX-2T (46). The highly soluble 42-kDa protein expressed by the resultant construct (pDA500) was purified on a glutathione-agarose affinity matrix, and the eluted protein was cleaved with thrombin to generate an approximate 17-kDa immunogen recognized by MAb 19G3 (Fig. 4). The phenylthiohydantoin-derivatized amino acids obtained from the 17-kDa cleavage product by 13 cycles of automated Edman degradation matched exactly the final 2 residues of GST (located on the carboxy-terminal side of the thrombin cleavage site [46]) and the first 11 residues predicted for the

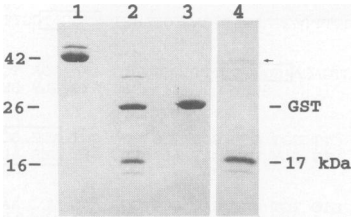


FIG. 4. Production of a nonlipidated 17-kDa immunogen from a GST fusion protein. Portions (2 μg each) of affinity-purified 17-kDa protein–GST fusion before (lane 1) and after (lane 2) cleavage with thrombin and purified GST (lane 3) were separated on an SDS–12.5% polyacrylamide gel and stained with Coomassie brilliant blue. The thrombin-cleaved material was immunoblotted with MAb 19G3 to identify the nonlipidated form of the 17-kDa immunogen (lane 4). Arrow indicates residual uncleaved fusion protein. Molecular mass standards in kilodaltons are shown at left.

mature 17-kDa immunogen (Fig. 3). For experiments described below, an additional GST fusion protein containing the mature portion of the antigenically unrelated *tpp15* gene product (36) was constructed. The N terminus of the nonlipidated 15-kDa cleavage product determined by protein sequencing exactly matched that determined previously by nucleotide sequence analysis (36).

**Lipid modification of the *tpp17* gene product.** To confirm that the *tpp17* gene product was lipid modified, *E. coli* DH5α harboring either pUC18 or pAE47 was labeled in vivo with [$^3$H]palmitate. The cell lysates were extracted with Triton X-114, phase partitioned, and immunoprecipitated with rabbit antisera directed against the 17-kDa protein–GST fusion protein. In order to control for both nonspecific binding and the anti-GST antibodies present in the antiserum (data not shown), extracts also were immunoprecipitated with an antiserum directed against the 15-kDa protein–GST fusion protein. Immunoblot analysis against *T. pallidum* whole-cell lysates confirmed the predicted specificities of both anti-GST fusion protein antisera (Fig. 5A). Interestingly, as with MAb 19G3, the anti-17-kDa protein–GST fusion antiserum also recognized higher-molecular-mass polypeptides, including the protein with an approximate molecular mass of 35 kDa (Fig. 5A). A radiolabeled immunoprecipitate was obtained only for the *E. coli* DH5α(pAE47) extract incubated

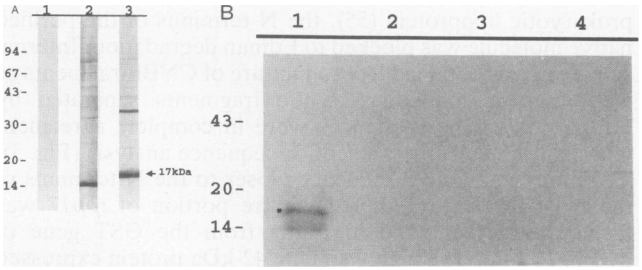


FIG. 5. Lipid modification of the recombinant 17-kDa membrane immunogen. (A) Immunoblot analysis against *T. pallidum* whole-cell lysates with rabbit antisera directed against purified GST (lane 1), 15-kDa protein–GST fusion (lane 2), and 17-kDa protein–GST fusion (lane 3). (B) Triton X-114 extracts of *E. coli* DH5α(pAE47) (lanes 1 and 2) and DH5α(pUC18) (lanes 3 and 4) radiolabeled with [$^3$H]palmitate were immunoprecipitated with rabbit antisera directed against the purified 17-kDa protein–GST fusion (lanes 1 and 3) or the 15-kDa protein–GST fusion (lanes 2 and 4). Molecular masses in kilodaltons are shown at left in both panels.

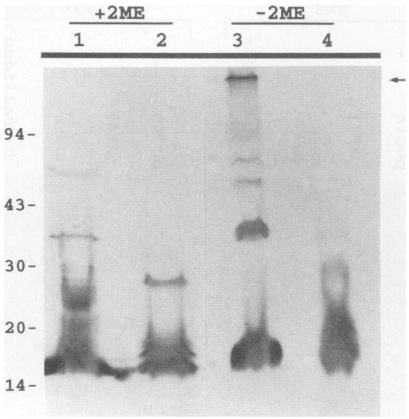


FIG. 6. Multimeric structure of the 17-kDa lipoprotein. Whole cells of *T. pallidum* (lanes 1 and 3) and *E. coli*(pAE47) (lanes 2 and 4) were boiled in final sample buffer in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 2-mercaptoethanol (2ME) prior to SDS-PAGE and immunoblotting with MAb 19G3. Arrow indicates the interface between stacking and separating gels. Molecular mass standards in kilodaltons are shown at left.

with the 17-kDa protein–GST fusion antiserum (Fig. 5B). Alignment of the autoradiograph in Fig. 5B with immunoblots of the same material confirmed that the most intensely labeled band was that of the mature 17-kDa immunogen (data not shown). The fainter bands with molecular masses greater or less than that of the mature immunogen appeared to correspond to precursor and breakdown products, respectively. Repeated attempts to immunoprecipitate the *T. pallidum* 17-kDa lipoprotein with either MAb 19G3 or the rabbit anti-17-kDa-protein–GST antiserum from treponemes intrinsically radiolabeled with [$^3$H]palmitate were unsuccessful (data not shown).

**Existence of disulfide-bonded multimeric forms of the native immunogen.** The possibility that the native immunogen exists in part as disulfide-bonded multimers was suggested by the presence of four cysteines (in addition to the lipid-modified residue of the N terminus) in the amino acid sequence deduced for the mature protein. To investigate this possibility, whole cells of *T. pallidum* and *E. coli* DH5α (pAE47) were solubilized in the presence or absence of 2-mercaptoethanol and then immunoblotted with MAb 19G3; gels were purposely overloaded to enhance visualization of low-abundance multimeric forms of the immunogen. As shown in Fig. 6, multimeric forms of the 17-kDa lipoprotein, including forms of the immunogen too large to enter the 12.5% polyacrylamide separating gel, were identified in unreduced *T. pallidum*. Interestingly, although reduction altered the SDS-PAGE mobility of the recombinant immunogen, multimeric forms comparable to those of the native immunogen were not identified (Fig. 6).

**Importance of covalently bound lipids for amphiphilicity and macrophage activation.** The discrepancy between the amphiphilicity of the 17-kDa immunogen (13, 38) and its apparent lack of membrane-spanning domains suggested that lipid modification was responsible for the molecule's hydrophobic character. To investigate this, the amphiphilicities of the native immunogen and the recombinant molecules expressed by *E. coli* DH5α(pAE47) and DH5α(pDA500) were compared by Triton X-114 phase partitioning. The native immunogen and that expressed by *E. coli* DH5α(pAE47) partitioned exclusively or predominantly into the detergent-

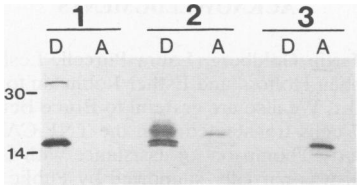


FIG. 7. Effect of lipid modification on amphiphilicity of the 17-kDa immunogen. *T. pallidum* (lanes 1) and *E. coli*(pAE47) (lanes 2) whole cells and recombinant 17-kDa immunogen cleaved from a GST fusion protein (lanes 3) were solubilized in 2% Triton X-114 and phase partitioned. The resulting detergent (D) and aqueous (A) phases were immunoblotted against MAb 19G3. Molecular mass markers in kilodaltons are shown at left.

enriched phase, while the nonlipidated immunogen, in contrast, was hydrophilic (Fig. 7).

We previously demonstrated that *T. pallidum* lipoproteins, including the 17-kDa immunogen, induced the synthesis of CAT in RAW 264.7 cells permanently transfected with a TNF-α promoter–CAT reporter construct (7, 40). The availability of the nonlipidated forms of these molecules enabled us to investigate the importance of lipid modification for cell activation and TNF-α biosynthesis. As previously demonstrated (40), purified native 17-kDa immunogen stimulated production of CAT, and this effect, unlike that of LPS, was not abrogated by polymyxin B. In contrast, the nonlipidated 17-kDa immunogen failed to induce CAT. To determine whether these findings were unique to the 17-kDa lipoprotein, parallel experiments with purified native 15-kDa *T. pallidum* lipoprotein (36) and its nonlipidated analog were performed. Once again, only the lipidated immunogen induced CAT biosynthesis (Fig. 8). The CAT response induced by the 15-kDa protein, which was stronger than that induced by the 17-kDa immunogen (Fig. 8), was consistent with earlier findings that spirochetal lipoproteins differ in their abilities to activate this murine macrophage-like cell line (40).

## DISCUSSION

The 17-kDa immunogen was discovered during immunoblot analysis of the humoral immune response to *T. pallidum* polypeptides in patients with acquired and experimental

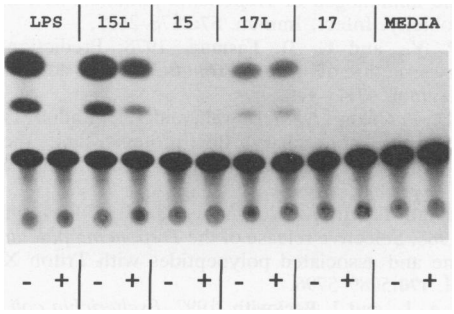


FIG. 8. Covalently bound lipids are required for macrophage activation by the 17-kDa immunogen. RAW 264.7 mouse macrophage cells transfected with a TNF-α–CAT reporter construct were incubated overnight with 1 μg each of *E. coli* LPS, purified native 15- or 17-kDa lipoproteins (15L and 17L, respectively), nonlipidated 15- or 17-kDa immunogen (15 and 17, respectively), or medium alone per ml in the presence (+) or absence (−) of polymyxin B (10 μg/ml).

syphilis (3, 4, 18, 19, 22). Our interest in the molecule emerged from efforts by ourselves and others to characterize *T. pallidum* membrane proteins by Triton X-114 phase partitioning (13, 38, 39). These studies not only highlighted the protein's extraordinary immunogenicity but also revealed that this protein is a relatively minor *T. pallidum* component (38). According to densitometric analysis of material separated by SDS-PAGE, it constitutes less than 2% of the DPPs (data not shown). The availability of a MAb (19G3) specific for this immunogen enabled its molecular characterization.

In previous studies, a 17-kDa lipoprotein was identified among the *T. pallidum* polypeptides intrinsically radiolabeled with $^3$H-fatty acids (9, 36). However, we were unable to confirm by immunoprecipitation with either MAbs or polyclonal antibodies that this lipoprotein was the same molecule as that recognized by MAb 19G3, presumably because of the native immunogen's limited abundance and the extremely low specific activities of [$^3$H]palmitate-labeled *T. pallidum* polypeptides. Nevertheless, the data indicating that the native and recombinant 17-kDa *T. pallidum* immunogens, defined by reactivity with MAb 19G3, are identical to the *T. pallidum* lipoprotein are compelling. First, we have demonstrated for a number of *T. pallidum* proteins that an excellent correlation exists between amphiphilicity (as determined by Triton X-114 phase partitioning), immunogenicity, and lipid modification (9, 10, 36, 48). Second, as expected for a prokaryotic lipoprotein, the native immunogen recognized by MAb 19G3 was blocked to Edman degradation (55). Most important, the deduced amino acid sequence of the cloned immunogen revealed a consensus tetrapeptide characteristic of prokaryotic lipoproteins (55), and radioimmunoprecipitation with a monospecific antiserum confirmed that the recombinant immunogen was lipid modified.

The large number of cysteine residues in the deduced amino acid sequence suggested that disulfide-bonded multimers of the 17-kDa immunogen might exist. Consistent with this, a number of multimeric forms were identified in *T. pallidum* whole cells solubilized in the absence of reducing agent. Interestingly, large multimers comparable to those of the native molecule were not identified in unreduced recombinant *E. coli* lysates. In *E. coli*, secretory proteins become disulfide bonded only after translocation and processing (14, 34). Thus, it is interesting to speculate that *T. pallidum* possesses a homolog to the recently described periplasmic disulfide-bond-forming protein of *E. coli* (6). Compared with other *T. pallidum* lipoproteins sequenced thus far (36, 45, 48, 54), the 17-kDa lipoprotein shows extensive disulfide bonding as a distinctive feature. The detection of higher-molecular-mass forms of both the native and recombinant immunogens after boiling and reduction of cell lysates suggested that strong noncovalent forces also contribute to the higher-order structure of the molecule. The existence in the 17-kDa immunogen of higher-order structure dependent on both disulfide and noncovalent bonds appears similar to previous findings made for the *T. pallidum* 4D antigen (16, 37).

In the past, we demonstrated the relationship between lipid modification and amphiphilicity of lipoproteins by comparing the Triton X-114 phase-partitioning behaviors of native and recombinant molecules and the corresponding polypeptides produced in vitro (10, 36, 48). In this study, we accomplished the same end by using a novel strategy, namely, generation of a nonlipidated fusion protein in combination with Triton X-114 phase partitioning. While this is not equivalent to proving that the covalently bound lipids provide a membrane anchor for the immunogen, such a

presumption seems credible given the hydrophilicity of the nonlipidated form of the molecule.

We previously demonstrated that the lipoprotein immunogens of *T. pallidum* induced murine macrophages to produce TNF-α and that the mechanism for this appeared to be distinct from that by which LPS activated TNF-α production by these same cells (40). We also reported that purified 47-kDa *T. pallidum* lipoprotein activates human vascular endothelial cells (42), another immune effector cell type highly relevant to syphilis pathogenesis (50). Production of a nonlipidated form of the 17-kDa immunogen has enabled us to demonstrate that lipid modification is responsible for the molecule's immunomodulatory activity as well as for its amphiphilicity. Similar findings for the 15-kDa immunogen (36) indicated that this generalization also applies to other *T. pallidum* lipoproteins. It could be argued that this conclusion is mitigated by the fact that the N termini of the nonlipidated proteins contained two amino acids not present in the native molecules. However, identical observations have been made by using synthetic pentapeptide and lipopentapeptide analogs that reproduce exactly the N termini of these molecules (unpublished data). Presently, the mechanism by which the lipoproteins activate immune cells is unclear. The requirement for covalently attached lipids suggests that the lipoprotein must insert via the lipids into the plasma membranes of effector cells before signal transduction occurs. Our findings also demonstrate the feasibility, in appropriate circumstances, of using the same genetically engineered constructs to investigate both the membrane topographies and the immunomodulatory effects of *T. pallidum* lipoproteins.

In recent years, combined ultrastructural and molecular analyses have provided insights into the composition and molecular architecture of *T. pallidum* that help elucidate clinical features of syphilis. One of the most important of these discoveries is that the major membrane immunogens of the pathogen, including the 17-kDa molecule characterized in this report, constitute a "family" of lipid-modified proteins (36, 48, 54). Although several of these immunogens have been described as being surface exposed (1, 23, 25, 32), our data are more consistent with their being anchored by N-terminal lipids to the periplasmic leaflets of the cytoplasmic and/or outer membranes (12, 38, 41). One major ramification of this newly proposed ultrastructural model (12) is that it explains the ability of virulent *T. pallidum* to resist binding antibodies directed against these major membrane immunogens (12, 21, 33, 47). At the same time, this model does not preclude the possibility that *T. pallidum* lipoproteins contribute to the induction of inflammation during syphilitic infection. Earlier, we reported that these molecules retain immunological activity even after extensive proteolysis (40); synthetic lipopentapeptides also reproduce the immunological effects of the native lipoproteins (unpublished data). Thus, lipoproteins could exert their biological activities even after spirochetes become degraded within host phagocytic cells (5, 28). The potential biological importance of the lipoproteins as immunomodulators during syphilitic infection is underscored by the fact that *T. pallidum* does not contain LPS (20, 39, 57). The evolution of these concepts concerning the relationship(s) between *T. pallidum* ultrastructure and syphilis pathogenesis demonstrates the value of integrating molecular studies of the bacterium with analysis of the immunopathologic mechanisms induced by the spirochete within the infected host.

## ACKNOWLEDGMENTS

We thank Martin Goldberg, Laura Purcell, Leslie Arndt, Lane Smothers, Stephen Holton, and Esther Robinson for their excellent technical support. We also are grateful to Bruce Beutler for providing RAW 264.7 cells transfected with the TNF-CAT reporter construct and to Patty Thompson for assistance with CAT assays.

This research was partially supported by Public Health Service grants AI-26756 and AI-16992 from the National Institute of Allergy and Infectious Diseases to J.D.R. and M.V.N., respectively; by grant-in-aid 91015470 from the American Heart Association to J.D.R.; and by grant I-0940 from the Robert A. Welch Foundation to M.V.N. and J.D.R. J.D.R. is the recipient of an Established Investigatorship Award from the American Heart Association.

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