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The 34-Kilodalton Membrane Immunogen of *Treponema pallidum* Is a Lipoprotein

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***Treponema pallidum* subsp. *pallidum* and *Escherichia coli* incorporated exogenous [^{3}H]palmitate into the 34-kilodalton (kDa) pathogen-specific antigen of *T. pallidum*. Radiolabeled fatty acid remained associated with the protein upon immunoprecipitation and after boiling in sodium dodecyl sulfate, acetone precipitation, and extensive extractions in organic solvents, suggesting that the fatty acid was covalently bound to the protein. Detection of [^{3}H]palmitate after alkaline and acid hydrolyses confirmed the identity of the incorporated label. Globomycin inhibited maturation of the recombinant 34-kDa antigen, suggesting that *E. coli* uses the lipoprotein-specific signal peptidase II to process the treponemal antigen. Globomycin also inhibited processing of the 34-kDa antigen, as well as the 44.5- and 15-kDa antigens, in *T. pallidum*, implying that *T. pallidum* also possesses the lipoprotein export pathway common to both gram-negative and gram-positive bacteria. Ethanol inhibited processing of the 34-kDa antigen in minicells, suggesting that the 34-kDa antigen normally is translocated through the cytoplasmic membrane. Comparison of the Triton X-114 phase partitioning behavior of the 34-kDa antigen produced either by minicells or by a cell-free translation system indicated that the covalent attachment of fatty acid conferred hydrophobic biochemical properties to the 34-kDa antigen, consistent with the hypothesis that the attached lipid anchors the 34-kDa antigen into the membrane.**

Ultrastructural characterization of the *Treponema pallidum* cell envelope is necessary for a complete understanding of the pathogenesis of syphilis because the molecular structure of the cell envelope probably contributes to bacterial invasion, dissemination, and subsequent evasion of host defenses (36). Treatment of intact *T. pallidum* with the nonionic detergent Triton X-114 followed by phase separation extracts a set of highly immunogenic proteins into the Triton X-114 detergent phase (8, 35). This set of detergent-phase proteins (DPPs) constitutes a major proportion of the immunogenic proteins of the *T. pallidum* cell envelope (8, 35). Recent metabolic labeling experiments with *T. pallidum* demonstrate that virtually the same set of immunogenic DPPs are modified by the covalent addition of fatty acid (3, 4).

The 34-kilodalton (kDa) antigen (15, 31, 39, 40, 44) cofractionates with the other *T. pallidum* integral membrane proteins into the Triton X-114 detergent phase (35, 39). However, because of the presence of other lipoproteins that comigrate with the 34-kDa antigen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it was not immediately apparent that the 34-kDa antigen also was a lipoprotein (3). Although the 34-kDa antigen is a membrane protein (35, 39, 40), hydropathy analysis of the protein sequence did not reveal any obvious transmembrane domains that could explain either its hydrophobic biochemical behavior or its membrane localization in both *Escherichia coli* and *T. pallidum* (39). The covalent addition of fatty acid to the 34-kDa antigen could provide a mechanism by which the antigen associates with the membrane. Furthermore, the sequence of the 34-kDa antigen contains the tetrapeptide Phe-Ser-Ala-Cys in its presumptive signal sequence (39), similar to the tetrapeptide Leu-X-Y-Cys consensus sequence required for modification of bacterial lipoproteins by diacylglycerol and subsequent cleavage by the lipoprotein-specific signal peptidase II (43, 47); this suggested that the 34-kDa antigen may be a lipoprotein. We, therefore, sought to determine whether the 34-kDa antigen is modified by the covalent addition of lipid in both *T. pallidum* and *E. coli* and whether the addition of the lipid could confer hydrophobicity.

MATERIALS AND METHODS

Bacteria and plasmids. *T. pallidum* subsp. *pallidum* (*T. pallidum*) (Nichols strain) was cultivated by serial intratesticular passage in New Zealand White rabbits (21). Plasmids were maintained in *E. coli* RR1 (30). *E. coli* ORN103 (4, 32) was used for minicell analysis. Recombinant plasmid pMN20, which encodes the *T. pallidum* 34-kDa antigen, contains a DNA insert of approximately 1.8 kilobases in the *Pst*I site of pBR322 (40). Plasmid pMSH209, which expresses the peptidoglycan-associated lipoprotein (PAL) of *Haemophilus influenzae* type b, was constructed by inserting the *Pst*I-*Bam*HI fragment encompassing the PAL gene (9) into the *Pst*I and *Bam*HI sites in the polylinker of plasmid pUC19. Plasmid pFRG100 (11), which expresses the heat-modifiable outer membrane protein, P1, of *H. influenzae* type b, was provided by Eric Hansen and Mark S. Hanson. Plasmid DNA was prepared as described earlier (40).

Antisera and MAbs. Monoclonal antibodies (MAbs) were generated, maintained, and purified as described previously (37). MAb 11E3 (immunoglobulin G2b[IgG2b]) is specific for the 47-kDa major integral membrane protein of *T. pallidum* (21). MAbs 3G9 (IgG2b) and 5C11 (IgG2b) are directed against the *T. pallidum* 34-kDa antigen (39). MAb 7C6 (IgG1), specific for the 38-kDa DPP of *T. pallidum*, was isolated by immunizing BALB/c mice with Triton X-114-extractable DPPs (35). Rabbit antisera to *T. pallidum* DPPs was described previously (35).

Chemicals. Triton X-114 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). L-[^{35}S]methionine (>800 Ci/mmol), L-[^{3}H]leucine (156 Ci/mmol), and

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L-3-phosphatidylethanolamine, 1,2-di[1-^{14}C]palmitoyl (110 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). [^{14}C]-molecular weight markers and 9,10(*n*)-[^{3}H]palmitate (30 Ci/mmol) were obtained from Dupont, NEN Research Products (Boston, Mass.). Rainbow molecular weight markers were purchased from Amersham. Molecular weight standards were phosphorylase *b* (92.5 kilodaltons [kDa]), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), and cytochrome *c* (12.3 kDa).

Radiolabeling of *T. pallidum* proteins. Treponemes at a concentration of 10^9 organisms per ml were radiolabeled in the medium of Stamm and Bassford (38) containing 100 μg of cycloheximide per ml (3, 35, 38). Either [^{3}H]palmitate or [^{35}S]methionine was added at a concentration of 0.25 mCi/ml (3). Nonradioactive methionine and cysteine were omitted from the medium when *T. pallidum* was labeled with [^{35}S]methionine. After 20 h of incubation at 34°C, the bacteria were extracted with 2% Triton X-114 in phosphate-buffered saline (10 mM sodium phosphate [pH 7.2], 140 mM NaCl) and washed as described previously (3, 35).

For globomycin inhibition experiments, globomycin dissolved in ethanol was added at a concentration of 100, 150, or 200 μg/ml. Final ethanol concentrations were less than 0.5%; ethanol was used in control experiments at the same concentrations. In several of the globomycin inhibition experiments, [^{35}S]methionine-labeled *T. pallidum* was lysed by boiling for 10 min in 1% SDS–20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. After two sequential centrifugations for 10 min at 13,000 × *g* to remove insoluble debris, portions of the supernatant were immunoprecipitated; the immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Radiolabeling of *E. coli*. Overnight cultures of *E. coli* RR1 (in L broth) containing either pBR322 or pMN20 were further diluted 1:100 in L broth (14, 24). When the A_{590} of the cultures reached 0.08 to 0.12, 250 μCi of 9,10(*n*)-[^{3}H]palmitate in ethanol (final ethanol concentration, 0.2%) was added, and growth was continued until the A_{590} reached 0.8 to 1.0. Radioactive cells were collected by centrifugation at 16,000 × *g* for 15 min. The cell pellet was washed once by centrifugation in phosphate-buffered saline. Cells were suspended in 1% SDS in 20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and lysed by heating at 100°C for 10 min. The insoluble residue was removed by two rounds of centrifugation at 13,000 × *g* for 10 min. Incorporation of [^{35}S]methionine was performed in a similar manner except that *E. coli* cells were grown in M9 medium supplemented with 0.2% glucose and one-quarter-strength methionine assay medium (29, 40). The proteins labeled with[^{35}S]methionine were precipitated by the addition of 10 volumes of cold (−70°C) acetone in preparation for SDS-PAGE analysis.

Delipidation of [^{3}H]palmitate-labeled *E. coli* proteins. Proteins were precipitated from lysates by the addition of 10 volumes of cold acetone and storage at −20°C for at least 1 h (3). The precipitate was extracted at least 10 times by adding 20 ml of chloroform-methanol (2:1 [vol/vol]) and by mixing vigorously (3, 14, 45). The residue was collected by centrifugation, suspended in 1% SDS (in distilled H_2O), and analyzed by SDS-PAGE, immunoprecipitation, or hydrolysis reactions. In some cases, delipidation was performed before and after immunoprecipitation.

Hydrolysis of [^{3}H]palmitate-labeled *E. coli* proteins. After immunoprecipitation with MAbs, the 34-kDa antigen was separated from the antigen-antibody-*Staphylococcus aureus* cell wall complex by boiling for 10 min in 1% SDS–20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, followed by centrifugation to remove *S. aureus* cells. SDS was removed by precipitating the [^{3}H]palmitate-labeled 34-kDa protein with acetone, followed by centrifugation. The protein precipitate was washed with acetone and then lyophilized. The dried precipitate was suspended in 1% SDS in distilled H_2O, and a sample was assayed by scintillation counting to determine radioactivity. Radiochemical purity was assayed by SDS-PAGE and fluorography. Aliquots containing equivalent amounts of radioactivity were used in hydrolysis experiments. For alkaline hydrolysis, the radiolabeled 34-kDa antigen was incubated in 0.5 ml of 0.1 N NaOH (in distilled H_2O) for 4 h at 37°C. The reaction mixture was then acidified by the addition of 6 N HCl. Negative control reaction mixtures were incubated in distilled H_2O for 4 h at 37°C. Acid hydrolysis was performed in 0.5 ml of 6 N HCl at 100°C for 4 h. Extractions were performed subsequent to each hydrolysis in chloroform-methanol (3). Fatty acid released by hydrolysis was assayed by liquid scintillation counting. The identity of the released fatty acid was confirmed as phenylacyl esters by high-pressure liquid chromatography as described by Chamberlain et al. (3).

Procaryotic DNA-directed in vitro translation assay. An *E. coli*-based cell-free translation assay system was obtained from Amersham and was used according to the instructions of the manufacturer (4, 39). To assess the amphiphilicity of the in vitro-derived product, 400 μl of cold 2% Triton X-114 in 20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA was added to the 35-μl final reaction volume; the sample was extracted for 4 h at 4°C, followed by removal of insoluble material, phase separation, washing, immunoprecipitation, and SDS-PAGE (4, 35). In reactions in which [^{3}H]palmitate or 1,2,di[1-^{14}C]palmitoyl phosphatidylethanolamine was the radioactive tracer, the radiolabel was first dried by lyophilization and then suspended in Triton X-100 mixed in dilution buffer (provided by the manufacturer) such that the Triton X-100 concentration in the final translation reaction mixture was 1%. Although Triton X-100 should not inhibit the cell-free translation system (49) or the activity of signal peptidases (42, 49), simultaneous control experiments under identical detergent conditions were performed using [^{35}S]methionine as the radiolabel. An amino acid chase solution was included in labeling reaction mixtures containing radioactive lipids to provide a complete set of amino acids for protein synthesis (4).

Expression in minicells. Minicells were prepared as described previously (4). Aliquots of prepared 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. Each minicell aliquot was suspended in 200 μl of M9 medium containing glucose and cycloserine. To allow degradation of endogenous mRNA, minicells were incubated at 37°C for 15 min prior to the addition of radioactive amino acids. L-[^{35}S]methionine was used for most experiments; L-[^{3}H]leucine was used to label minicells that expressed the PAL of *H. influenzae* type b because the mature lipoprotein contains no methionine residues (9). Both amino acids were used at a final concentration of 50 μCi/ml; incubations proceeded for 30 min. For experiments involving globomycin inhibition of processing, globomycin (dissolved in ethanol) at a final concentration of 100 μg/ml was added 5 min prior to the addition of radioactive amino acids. The final ethanol concentration was 1%; control experiments without globomycin used ethanol at the same concentration. For experiments involving ethanol inhibition of processing, ethanol at final concentrations of 0, 2, 4, 6, 8, and 10%

(vol/vol) was added immediately prior to the addition of radioactive tracer. For phase separation experiments with minicells, the washed minicell pellet was suspended in 2% Triton X-114 in 20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and extracted for 4 h at 4°C prior to phase separation (4, 35). For fatty acid labeling experiments with minicells, L broth containing 20 μg of cycloserine per ml was used in place of the M9-based medium. One hundred microcuries of [^{3}H]palmitate was used to label each minicell aliquot. The ethanol solvent was first removed by lyophilization, and the dried [^{3}H]palmitate was incubated in L broth for 2 h prior to the addition of washed minicells. The minicells were incubated for 1 h at 37°C. After incubation, the labeled minicells were washed by centrifugation to remove excess label. Samples were then processed for either immunoprecipitation or SDS-PAGE and fluorography.

Radioimmunoprecipitations. Radioimmunoprecipitations were performed as described previously (3, 4, 21, 29, 40). Aliquots containing radiolabeled antigens were diluted by the addition of 1 ml of phosphate-buffered saline containing 1% Triton X-100. For MAb 7C6 (IgG1), rabbit anti-mouse IgG was added as a bridging antibody.

SDS-PAGE and fluorography. SDS-PAGE was performed using 4% stacking and 12.5% separating gels as described previously (21, 40). All samples were boiled and reduced (21, 40). All samples that contained Triton X-114 were acetone precipitated prior to SDS-PAGE analysis. Sodium salicylate was used for fluorographic enhancement of samples radiolabeled only with [^{35}S]methionine (3, 4). Gels that contained molecules labeled with either [^{3}H]palmitate alone or both [^{35}S]methionine and [^{3}H]palmitate were fluorographed by immersion in En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) prior to being dried and exposed to Fuji X-ray film.

RESULTS

***T. pallidum* acylates the 34-kDa antigen.** The 34-kDa antigen displays a characteristic diffuse electrophoretic mobility in one-dimensional SDS-PAGE and, therefore, can be difficult to distinguish from other comigrating *T. pallidum* lipoproteins (3, 31, 35). Immunoprecipitation experiments were necessary to identify the 34-kDa antigen in Triton X-114 detergent-phase extracts of *T. pallidum* that had incorporated [^{3}H]palmitate. MAb 3G9 (Fig. 1, lane 3) specifically identified the 34-kDa antigen as a lipoprotein in *T. pallidum*; control immunoprecipitations of the 47- and 38-kDa DPPs confirmed that these two proteins also are lipoproteins in *T. pallidum* (Fig. 1, lanes 1 and 2, respectively) (3). The *T. pallidum* proteins that were labeled by [^{3}H]palmitate are identical to those described by Chamberlain et al. (3).

***E. coli* acylates the 34-kDa antigen of *T. pallidum*.** *E. coli* RR1 containing recombinant plasmid pMN20 incorporated exogenous [^{3}H]palmitate into the 34-kDa treponemal antigen (Fig. 2); the 34-kDa antigen was identified easily in SDS-PAGE profiles of whole-cell lysates from palmitate-labeled *E. coli* (Fig. 2B, lane 2) because of its characteristic electrophoretic profile and the absence of comigrating *E. coli* lipoproteins. Moreover, MAb 3G9 specifically immunoprecipitated the [^{3}H]palmitate-labeled 34-kDa antigen from whole-cell lysates (Fig. 2C, lane 1), confirming the identity of the antigen. It is noteworthy that *E. coli* incorporated exogenous [^{3}H]palmitate into a limited subset (Fig. 2B) of the total proteins that are synthesized during bacterial growth (Fig. 2A). Furthermore, the moieties labeled by radioactive palmitate corresponded in number and apparent molecular masses to the previously described lipoproteins of *E. coli* (Fig. 2B) (18). The radioactivity associated with the immunoprecipitated 34-kDa antigen was identified by high-pressure liquid chromatography as [^{3}H]palmitate after both alkaline and acid hydrolyses (3; data not shown).

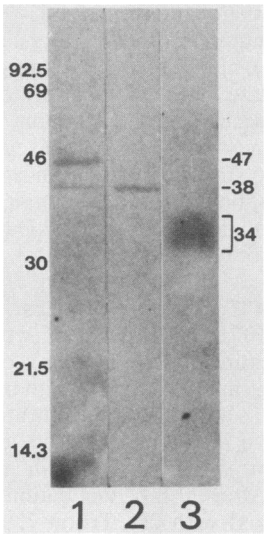

FIG. 1. Radioimmunoprecipitation analysis of [^{3}H]palmitate-labeled 47-, 38-, and 34-kDa DPPs of *T. pallidum*. Lanes 1, 2, and 3, MAbs 11E3, 7C6, and 3G9, respectively. Molecular size markers are on the left. Apparent molecular masses are in kilodaltons.

Globomycin inhibits processing of the 34-kDa antigen in *E. coli*. The tetrapeptide sequence Phe-Ser-Ala-Cys in the deduced amino acid sequence of the 34-kDa antigen (39) is similar to the Leu-X-Y-Cys consensus sequence (43, 47, 48) that is necessary for cleavage of lipoprotein precursors by signal peptidase II. Treatment of *E. coli* minicells containing plasmid pMN20 with globomycin, a specific inhibitor of signal peptidase II (10, 17, 42), at 100 μg/ml, inhibited the conversion of the precursor to the mature form of the 34-kDa antigen (Fig. 3, lanes 1 and 2). Parallel globomycin treatment of *E. coli* minicells that express PAL of *H. influenzae* type b (9) demonstrated that globomycin inhibited the maturation of PAL but had no effect on the processing of the nonlipoprotein β-lactamase encoded by the pUC19 cloning vector (Fig. 3, lanes 3 and 4).

Globomycin inhibits processing of the 34-kDa antigen in *T. pallidum*. *T. pallidum* was treated with globomycin to test whether *T. pallidum* and *E. coli* process the 34-kDa antigen in a similar manner. Incubation of *T. pallidum* with or without globomycin, followed by immunoprecipitation with MAb 3G9, showed that treatment of *T. pallidum* with 200 μg of globomycin per ml (Fig. 4, lane 2) inhibited the appearance of the mature form of the 34-kDa antigen (Fig. 4, lane 1). At lower concentrations of globomycin, inhibition was incomplete (data not shown). Globomycin at 100 μg/ml, however, appeared to inhibit the processing of the 44.5- and 15-kDa DPPs but had no apparent effect on the maturation of the 47-, 38-, and 17-kDa lipoproteins (3) (data not shown). Relatively high concentrations of globomycin also seemed to cause a generalized inhibition of protein synthesis in *T. pallidum* (Fig. 4, lane 2), an effect which also occurs in *E. coli* (17). The higher-molecular-weight bands in Fig. 4 also were present in immunoprecipitations with negative control antibodies (data not shown).

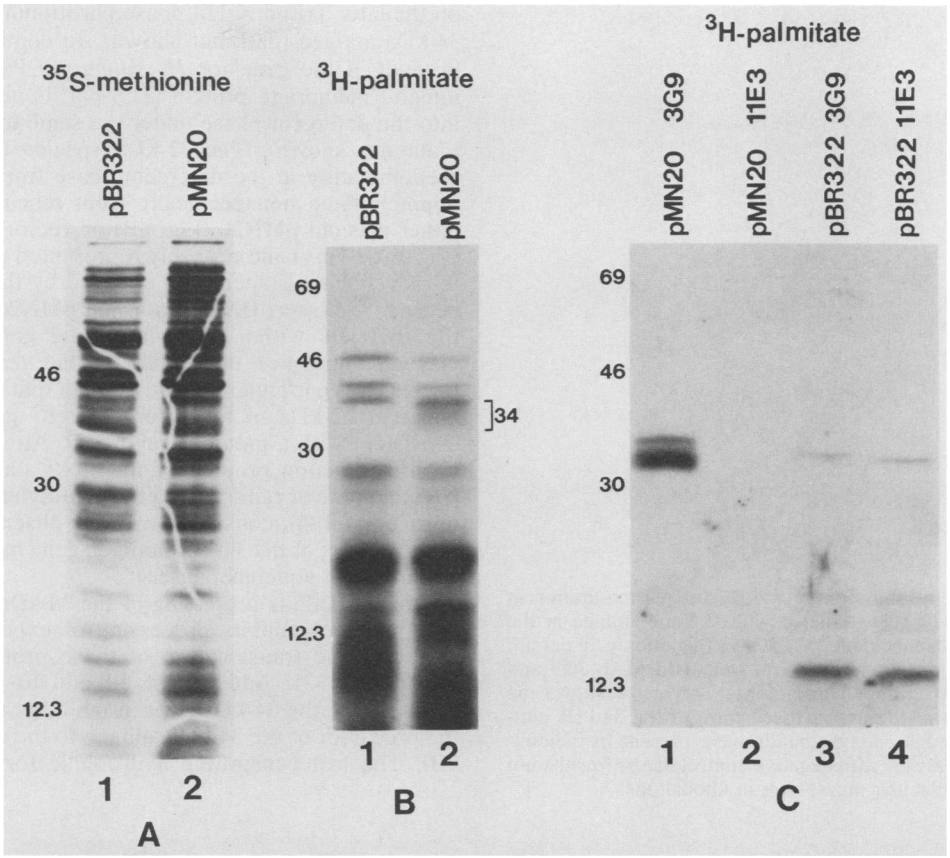

FIG. 2. SDS-PAGE of radiolabeled 34-kDa antigen in *E. coli*. (A) *E. coli* RR1 containing plasmid pBR322 (lane 1) or pMN20 (lane 2) was metabolically labeled with [^{35}S]methionine. (B) *E. coli* RR1 containing plasmid pBR322 (lane 1) or pMN20 (lane 2) was intrinsicially labeled with [^{3}H]palmitate. (C) Radioimmunoprecipitation of [^{3}H]palmitate-labeled *E. coli* RR1 containing pMN20 (lanes 1 and 2) or pBR322 (lanes 3 and 4) with 34-kDa-antigen-specific MAb 3G9 (lanes 1 and 3) and negative control MAb 11E3 (lanes 2 and 4). Apparent molecular masses are in kilodaltons.

Covalent addition of fatty acids confers amphiphilicity to the 34-kDa antigen. Phase partitioning with Triton X-114 was used to compare the amphiphilic behavior of the 34-kDa antigen produced by *E. coli* minicells with that of the protein produced in an *E. coli*-derived cell-free translation system (4, 34). Immunoprecipitations using MAb 5C11 and antisera directed against the DPPs confirmed that the 34-kDa antigen synthesized in minicells partitioned predominantly into the detergent phase after extraction of minicells with Triton X-114 (Fig. 5B, lanes 1 and 5). The molecular species that partitioned into the aqueous phase (Fig. 5B, lanes 2 and 6, asterisk) was derived from the 34-kDa-antigen gene, because it was specifically immunoprecipitated by MAb 5C11 (Fig. 5A); however, this species was probably an artifact of the recombinant *E. coli* host because no equivalent entity is observed in analyses of *T. pallidum* (31, 35, 39, 40). This hydrophilic molecular species was not labeled by [^{3}H]palmitate in *E. coli* whole cells (Fig. 2C). Minicells containing pMN20 also incorporated [^{3}H]palmitate specifically into the 34-kDa antigen (data not shown).

Immunoprecipitation analysis of the products of a cell-free DNA-directed translation system demonstrated that the primary product of the 34-kDa-antigen gene partitioned almost exclusively into the aqueous phase after phase separation of the reaction products with Triton X-114 (Fig. 6B, lanes 5 and 6). Inclusion of 1% Triton X-100 in the synthesis reaction mixtures did not inhibit protein synthesis and had no effect

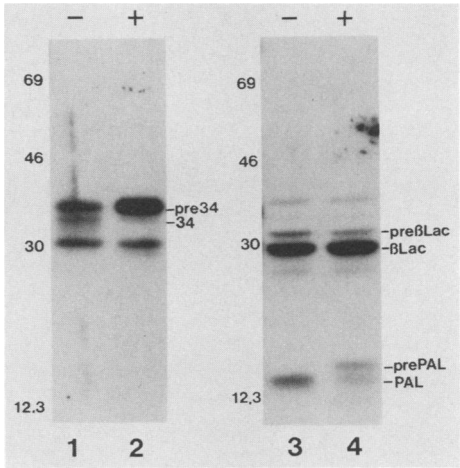

FIG. 3. Globomycin inhibition of processing in *E. coli* minicells. *E. coli* minicells containing pMN20 were labeled with [^{35}S]methionine in the presence (lane 2) or absence (lane 1) of globomycin, followed by immunoprecipitation with 34-kDa-antigen-specific MAb 3G9, SDS-PAGE, and fluorography. *E. coli* minicells containing pMSH209 were labeled with [^{3}H]leucine in the presence (lane 4) or absence (lane 3) of globomycin, followed by SDS-PAGE and fluorography. PAL, Globomycin-sensitive peptidoglycan-associated lipoprotein of *H. influenzae* type b; βLac, vector (pUC19)-encoded β-lactamase. Apparent molecular masses are in kilodaltons.

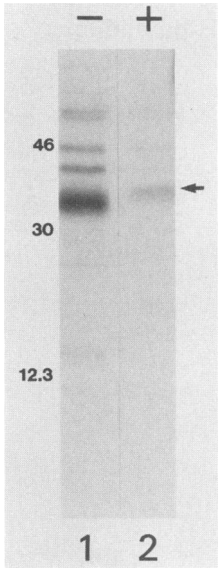

FIG. 4. Globomycin inhibition of 34-kDa-antigen maturation in *T. pallidum*. *T. pallidum* was labeled with [^{35}S]methionine in the presence (lane 2) or absence (lane 1) of 200 μg of globomycin per ml. The 34-kDa antigen was immunoprecipitated with MAb 3G9 and analyzed by SDS-PAGE and fluorography. The arrow shows the approximate location of the unprocessed form of the 34-kDa antigen. The higher-molecular-weight bands were present in radioimmunoprecipitation analyses with negative control MAbs (results not shown). Apparent molecular masses are in kilodaltons.

on the later Triton X-114 phase partitioning behavior of the 34-kDa antigen (data not shown). In contrast, the primary product of the gene for *H. influenzae* P1, a conventional integral membrane protein (11), partitioned predominantly into the detergent phase under the same in vitro conditions (data not shown). The 32-kDa species that was present predominantly in the detergent-phase fractions was immunoprecipitated nonspecifically from reactions primed with either plasmid pMN20 (Fig. 6B) or vector plasmid pBR322 (Fig. 6A). This band probably represented the product of the tetracycline resistance gene encoded by the pBR322 vector because the insert DNA of plasmid pMN20 was cloned into the *Pst*I site within the β-lactamase gene. Furthermore, plasmid subclones that interrupt the *tetC* gene did not express this particular 32-kDa product (data not shown). The apparent 32-kDa mobility for the *tetC* gene product was consistent with a previous report (7). Attempts to label the in vitro reaction product of the 34-kDa-antigen gene with [^{3}H]palmitate or radioactive phosphatidylethanolamine were unsuccessful (4), consistent with the observation that the in vitro product of the 34-kDa-antigen gene partitioned into the Triton X-114 aqueous phase.

Ethanol inhibits processing of the 34-kDa antigen in minicells. Ethanol inhibits processing of exported proteins by inhibiting the translocation of these proteins through the membrane (33). Addition of ethanol to *E. coli* minicells synthesizing the 34-kDa antigen inhibited the conversion of the precursor of the 34-kDa antigen to the mature form (Fig. 7B). The faster-migrating hydrophilic form of the 34-kDa

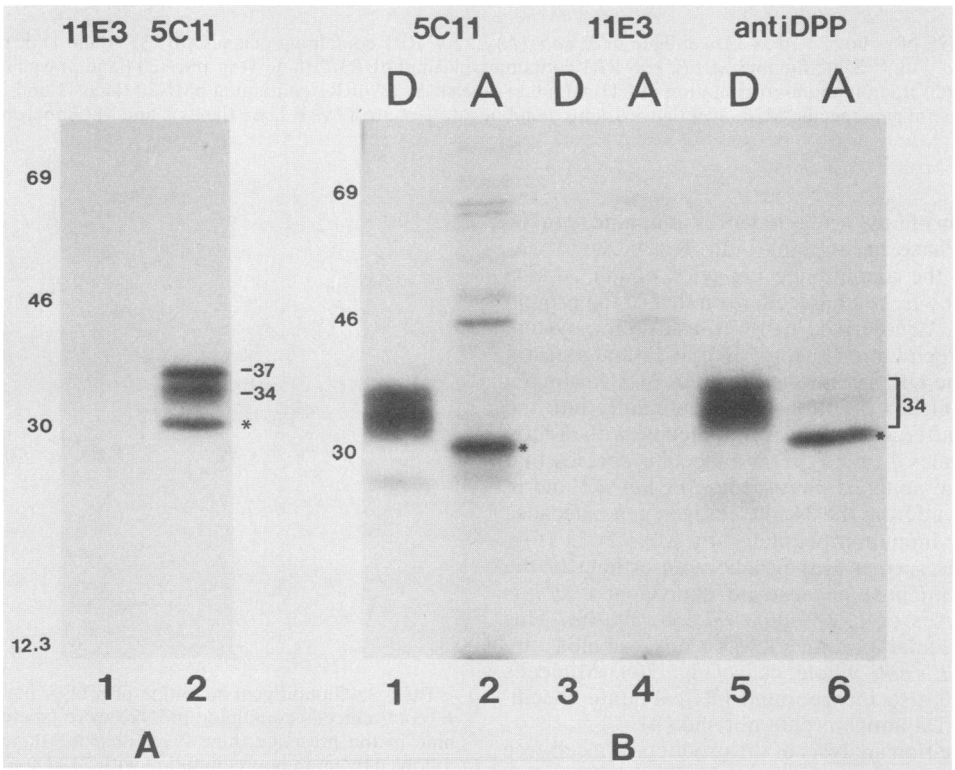

FIG. 5. Phase partitioning and immunoprecipitation analysis of 34-kDa antigen expressed in *E. coli* minicells. (A) *E. coli* minicells containing pMN20 were labeled with [^{35}S]methionine and immunoprecipitated with either MAb 5C11 (lane 2) or negative control MAb 11E3 (lane 1). (B) *E. coli* minicells containing pMN20 were labeled with [^{35}S]methionine, phase partitioned with Triton X-114, and immunoprecipitated with MAb 5C11 (lanes 1 and 2), MAb 11E3 (lanes 3 and 4), and antisera to the *T. pallidum* DPPs (lanes 5 and 6), followed by SDS-PAGE and fluorography. The asterisks indicate a hydrophilic derivative of the 34-kDa-antigen. D, Detergent phase; A, aqueous phase. Apparent molecular masses are kilodaltons.

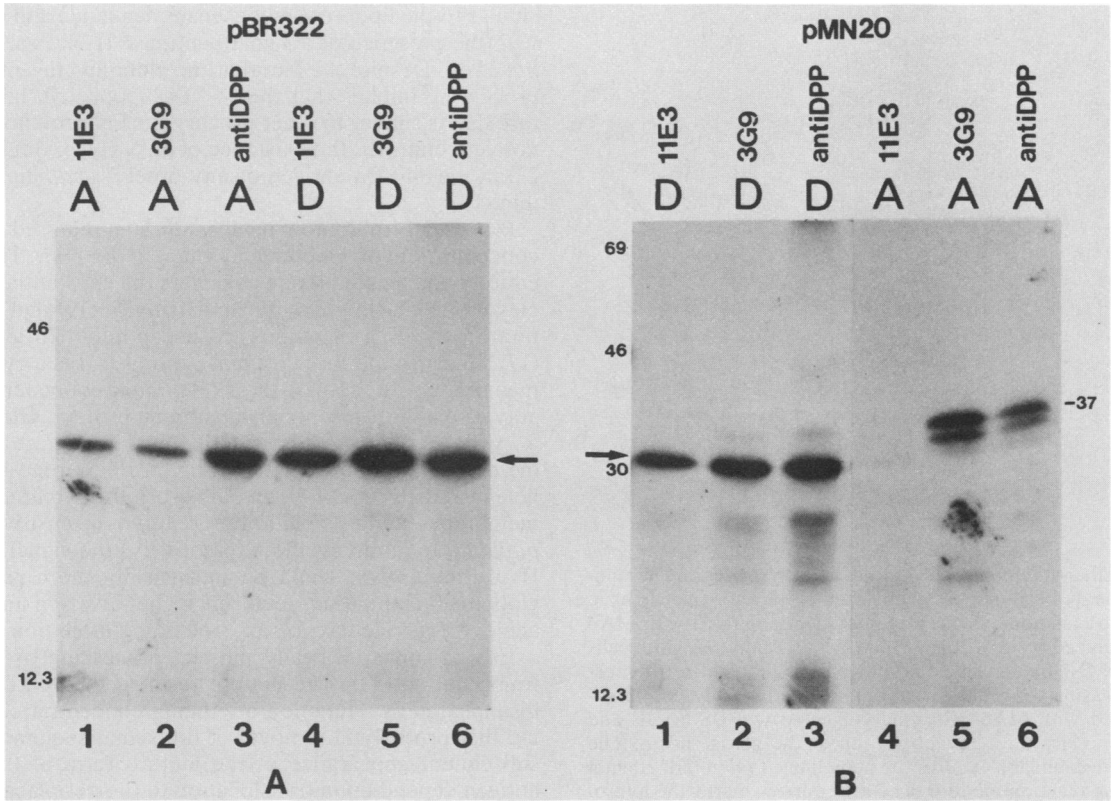

FIG. 6. Phase partitioning and immunoprecipitation of in vitro-translated 34-kDa antigen. The in vitro products of pBR322 (A) and p MN20 (B) were labeled with [^{35}S]methionine, phase partitioned with Triton X-114, and immunoprecipitated with MAb 3G9 (lanes 2 and 5), MAb 11E3 (lanes 1 and 4), and antisera to the DPPs (lanes 3 and 6), followed by SDS-PAGE and fluorography. The arrow indicates a vector-encoded product. A, Aqueous phase; D, detergent phase. Apparent molecular masses are in kilodaltons.

antigen (Fig. 7B, lower arrow) also disappeared as processing was inhibited at the higher ethanol concentrations (Fig. 7B). Simultaneous with the disappearance of the translocated forms of the 34-kDa antigen at high ethanol concentrations was the appearance of an immunoprecipitable product with a higher apparent molecular mass (Fig. 7B, 8 and 10% ethanol, upper arrow) than those of the primary products synthesized by minicells or in the cell-free in vitro translation system (Fig. 7A). Maturation of the pUC19-encoded β-lactamase (Fig. 7C) appeared to be slightly more sensitive to ethanol inhibition than was maturation of the 34-kDa antigen (Fig. 7B).

DISCUSSION

The data presented here demonstrate that the 34-kDa integral membrane protein of *T. pallidum* is a lipoprotein. Both *T. pallidum* and *E. coli* incorporated exogenous radioactive fatty acid into the 34-kDa protein. The radioactive fatty acid remained associated with the 34-kDa antigen after repeated boiling in SDS and extensive extractions with organic solvents and was released only after alkaline or acid hydrolysis, demonstrating that the fatty acid is covalently associated with the 34-kDa antigen.

The 34-kDa product, as expressed in *E. coli*, appears to be a typical bacterial lipoprotein. Most of the previously described bacterial lipoproteins have structures that are similar to the murein lipoprotein of *E. coli* (13). They are synthesized as higher-molecular-weight precursors that are subsequently modified by the covalent addition of a diacylglycerol (containing two alkali-labile, esterified fatty acids) in a thioether linkage to a cysteine residue in the polypeptide chain (43, 47). The consensus sequence for the addition of the diacylglycerol and for the subsequent cleavage of the signal peptide by signal peptidase II is Leu-X-Y-Cys, where residues X and Y generally are amino acids with small, nonpolar side chains (43, 47, 48). After proteolytic removal of the signal peptide, another fatty acid is attached in an amide linkage (acid labile) to the amino group of the N-terminal cysteine (13, 43, 47). We suggested previously (39) that the 34-kDa lipoprotein has several features that imply that it is processed by the same pathway as other procaryotic lipoproteins. The primary translation product has a higher apparent molecular weight than the mature product, and pulse-chase processing experiments indicate that the primary product matures to a form which shows the characteristic electrophoretic mobility of the native molecule. The deduced protein sequence of the 34-kDa antigen predicts that the antigen also contains an amino-terminal signal sequence (39); the tetrapeptide Phe-Ser-Ala-Cys near the predicted cleavage site of the signal peptide is structurally similar to the Leu-X-Y-Cys consensus sequence for bacterial lipoproteins (43, 47, 48). Although no previously reported lipoproteins have phenylalanine in the −3 position from the cleavage site, the requirement for leucine in this position is not absolute. Substitutions with nonpolar residues such as threonine (41), serine (27), valine (9, 46), and isoleucine (6) have been described (48). Treatment of *E. coli*-derived minicells with globomycin, a cyclic peptide which specifi-

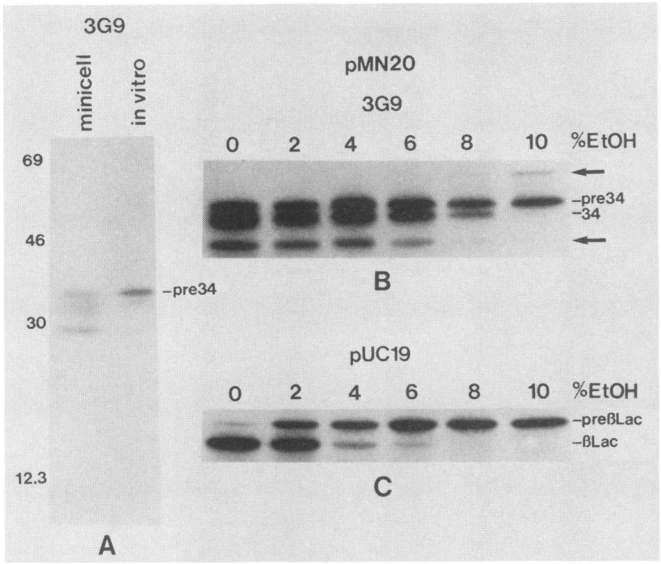

FIG. 7. Ethanol inhibition of 34-kDa-antigen processing in minicells. (A) The 34-kDa antigen synthesized either by minicells or in the in vitro translation system was immunoprecipitated with MAb 3G9 and analyzed by SDS-PAGE and fluorography. (B) Minicells containing pMN20 were labeled with [^{35}S]methionine in the presence of increasing concentrations of ethanol (EtOH), lysed, immunoprecipitated with MAb 3G9, and subjected to SDS-PAGE and fluorography. Only the relevant portion of the gel is shown. The number above each lane denotes the percentage (vol/vol) of ethanol in the labeling reaction mixture. The lower arrow marks the hydrophilic derivative of the 34-kDa antigen; the upper arrow denotes a possible maturational intermediate of the 34-kDa immunogen. (C) Minicells containing pUC19 and expressing β-lactamase (βLac) were labeled with [^{35}S]methionine in the presence of different concentrations of ethanol (EtOH), lysed, and analyzed by SDS-PAGE and fluorography. Ethanol concentrations are indicated as in panel B. Only the relevant portion of the gel is shown

cally inhibits the lipoprotein-specific signal peptidase II (10, 17, 26, 47), abolished the maturation of the 34-kDa antigen. Because signal peptidase II has an absolute requirement for the covalent addition of a diacylglycerol prior to cleavage of the signal peptide (43, 47), inhibition of processing by globomycin strongly suggests that *E. coli* processes the 34-kDa lipoprotein by the same pathway that it processes its own lipoproteins. The globomycin sensitivity of 34-kDa-antigen maturation also suggests that the fatty acid is attached to the protein by means of thioether-linked diacylglycerol (47) and that phenylalanine is an acceptable substitute for leucine in the signal peptidase II consensus sequence. The recovery of ester-linked and amide-linked radioactive palmitate after alkaline and subsequent acid hydrolyses, respectively, of the recombinant 34-kDa antigen provides further support for the hypothesis that the 34-kDa antigen is a conventional bacterial lipoprotein.

The globomycin sensitivity of 34-kDa-antigen processing also implies that *T. pallidum* possesses a lipoprotein export system analogous to that of *E. coli*. The recent observation that globomycin also inhibits the processing of both the cloned and native *T. pallidum* 15-kDa membrane antigens, as well as the 44.5-kDa DPP, provides additional evidence for the existence of this pathway in *T. pallidum* (unpublished results). The 44.5-kDa DPP, using the nomenclature of Norris et al. (31), is probably identical to TmpA (12). The sequence of TmpA contains the tetrapeptide Leu-Gly-Ser-Cys near the postulated signal peptide cleavage site (12), similar to the lipoprotein consensus sequence and consistent with the presence of a signal peptidase II-like enzyme in *T. pallidum*. Despite the fact that the globomycin sensitivity of processing implies that the 34-kDa lipoprotein has a structure that is similar to other procaryotic lipoproteins, we have not yet confirmed the existence of an *S*-glycerylcysteine (14, 23) in the 34-kDa antigen or any other *T. pallidum* lipoprotein.

Processing inhibition in *T. pallidum* required a greater concentration of globomycin than in *E. coli*. This is not entirely unexpected if one considers the substantial interspecies variability in globomycin sensitivity (19) and the variation among *E. coli* strains in susceptibility to globomycin (17). Because the *E. coli* signal peptidase II is a cytoplasmic membrane protein (42), the *T. pallidum* equivalent is probably also an intrinsic inner membrane protein. Globomycin, a cyclic peptide antibiotic (26), must penetrate the outer membrane before it can inhibit lipoprotein processing. Inefficient transport of globomycin across the *T. pallidum* outer membrane could explain the relative insensitivity of *T. pallidum* to globomycin. Alternatively, the signal peptidase II of *T. pallidum* could be intrinsically more resistant to globomycin inhibition, requiring higher levels of antibiotic to achieve equivalent levels of processing inhibition.

Ethanol nonspecifically inhibits processing by inhibiting translocation (33). The mature form of the 34-kDa antigen disappeared as a function of ethanol concentration, indicating that proteolytic removal of the signal sequence and the subsequent appearance of the mature form of the 34-kDa antigen depend upon translocation to the periplasmic side of the cytoplasmic membrane. In combination with the globomycin inhibition data, this observation provides convincing evidence that the 34-kDa antigen also is exported through the *T. pallidum* cytoplasmic membrane. Because the hydrophilic product of the 34-kDa-antigen gene was seen only in *E. coli* cell systems, it was probably an artifact of the recombinant host. However, consideration of the behavior of this molecular species can clarify certain features of the parent molecule. The hydrophilic, lower-molecular-weight form of the 34-kDa antigen, generated by *E. coli* minicells, also disappeared as the ethanol concentration increased, implying that this form also appears after translocation to the periplasmic space. It is likely that a periplasmic protease releases it from the membrane-bound form of the 34-kDa antigen. The soluble extracellular form of the *Bacillus licheniformis* penicillinase is generated by proteolytic cleavage in a similar manner (20, 28). The absence of fatty acid in this hydrophilic 34-kDa variant also explains its partitioning into the aqueous phase after extraction with Triton X-114 (see below). Treatment of minicells with ethanol also resulted in the appearance of a form of the 34-kDa antigen that migrated at a higher apparent molecular mass than the primary product. Because this form was immunoprecipitable with specific MAbs, it is an altered product of the 34-kDa-antigen gene. The nature of the alteration is unclear, but it may represent a form of the 34-kDa antigen modified by the addition of glycerol and fatty acids, and as such, an intermediate in the maturation of the 34-kDa antigen.

Analysis of the peptide sequence of the 34-kDa antigen suggested that the protein is relatively hydrophilic (39). The products synthesized in the in vitro translation system partitioned almost exclusively into the aqueous phase, suggesting that the hydrophilicity of the primary product prevents incorporation of the 34-kDa antigen into the Triton X-114 detergent phase. The failure to detect incorporated fatty acid in the 34-kDa antigen synthesized by the in vitro

translation system implies that the primary product is hydrophilic because it lacks covalently attached fatty acid. In contrast to the behavior of the in vitro-synthesized product, the minicell-derived 34-kDa antigen partitioned into the Triton X-114 detergent phase, consistent with the results for the antigen produced by recombinant *E. coli* whole cells (39). Both *E. coli* minicells and whole cells produced fatty acid-conjugated 34-kDa antigen; the hydrophilic form of the 34-kDa antigen produced by minicells was not labeled by [^{3}H]palmitate. Furthermore, the [^{3}H]palmitate-labeled 34-kDa antigen produced by *T. pallidum* was specifically immunoprecipitable from the Triton X-114 detergent phase. Thus, the ability of the 34-kDa antigen to partition into the Triton X-114 detergent phase correlates with the presence of covalently attached fatty acid.

The amino-terminal lipid is the means by which most procaryotic lipoproteins remain associated with the membrane. By conferring hydrophobicity on a polypeptide that is primarily hydrophilic, the covalently attached fatty acids probably anchor the 34-kDa antigen into both *E. coli* and *T. pallidum* membranes. The previous finding (4, 5, 16) that the hydrophobicity and membrane localization of the 47-kDa antigen depend upon the presence of covalently attached lipid supports this contention. The presence of fatty acid on all of the highly immunogenic membrane proteins of *T. pallidum* (3) further suggests that fatty acid is responsible for the membrane location of all of these extremely hydrophobic proteins (4).

The DPPs described by Radolf et al. (35), including the 34-kDa antigen, are major immunogenic proteins of the *T. pallidum* cell envelope. The observation that the immunogenic DPPs also are lipoproteins raises the intriguing possibility that the modification of these proteins by the addition of lipid contributes significantly to their immunogenicity. In this context, it is relevant to note that the murein lipoprotein of *E. coli* acts as a polyclonal B-cell activator and an adjuvant (1, 2, 22, 25). Furthermore, the portion of the lipoprotein molecule responsible for B-cell activation is the N-terminal lipopeptide; the presence of the lipids is essential for immunomodulation (25). The observation that the highly immunogenic DPPs are lipoproteins, coupled with the immunomodulatory properties of lipopeptides, suggests that the covalently attached fatty acids are at least partially responsible for the strong immune response directed against these antigens.

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