Acylation of the 47-Kilodalton Major Membrane Immunogen of *Treponema pallidum* Determines Its Hydrophobicity

NEAL R. CHAMBERLAIN,¹ LYNN DEOGNY,² CLIVE SLAUGHTER,² JUSTIN D. RADOLF,³
AND MICHAEL V. NORGARD^{1*}

Departments of Microbiology¹ and Internal Medicine³ and Howard Hughes Medical Institute,² University of Texas
Southwestern Medical Center at Dallas, Dallas, Texas 75235

Received 20 March 1989/Accepted 15 June 1989

The 47-kilodalton (kDa) major integral membrane immunogen of *Treponema pallidum* was recently found to be a proteolipid. Similar two-dimensional electrophoretic mobilities and common hydrophobic properties displayed by the native (*T. pallidum*) and recombinant (*Escherichia coli*) 47-kDa antigens suggested that the recombinant antigen also possesses covalently bound lipid. Both intact *E. coli* and *E. coli* minicells acylated the 47-kDa antigen; immunoprecipitation with a monoclonal antibody specific for the 47-kDa immunogen supported the contention that the acylated product of *E. coli* corresponds to the cloned *T. pallidum* antigen. Triton X-114 phase partitioning was used to compare the relative hydrophobicities of 47-kDa molecules synthesized by in vitro and in vivo protein translation systems. The products synthesized by *T. pallidum*, intact *E. coli*, or *E. coli* minicells were hydrophobic, while the protein synthesized in an *E. coli* cell-free translation system was hydrophilic. Processing experiments with *E. coli* suggested that the primary gene translation product of the protein is not synthesized in a precursor form, unlike other bacterial proteolipids. These results indicate that the hydrophobicity of the 47-kDa integral membrane protein is conferred substantially by the covalently attached lipid(s). The biochemical similarities between the native and recombinant 47-kDa proteolipids will provide a foundation for future investigations into the structure and immunogenicity of this integral membrane protein of *T. pallidum*.

Treponema pallidum subsp. *pallidum* is the etiologic agent of venereal syphilis, a chronic sexually transmitted disease of humans. Many aspects of the complex host-spirochete interaction(s) that characterizes syphilis are poorly understood. For example, it is well known that viable organisms may persist for years in both humans and experimental animals despite vigorous humoral and cellular immune responses (5, 14, 32, 38). Evidence that the immune system is able to control the infection is provided by the finding that rabbits and approximately one-third of untreated individuals never develop manifestations of tertiary syphilis (6, 32, 38). Furthermore, protective immunity ultimately can develop, inasmuch as both patients and animals with untreated latent infections may become resistant to exogenous challenge (32, 38). Investigation of the immunogens and the potential virulence factors operative in the immunopathogenesis of syphilis has been hampered by the requirement for continuous cultivation of *T. pallidum* in rabbits or other suitable mammalian hosts. Recently, this limitation has been circumvented, at least in part, by the expression of *T. pallidum* DNA in *Escherichia coli* (34).

One treponemal molecule of considerable interest has been the 47-kilodalton (kDa) antigen (4, 11, 12). Investigation of this protein was prompted by recognition in several laboratories of its intense immunogenicity, its relative abundance, and its pathogen specificity (for a review, see reference 20). Studies have established the amphiphilic properties of the 47-kDa antigen (4, 7, 27); both the native treponemal and the recombinant DNA-derived forms of the protein require detergents for solubilization (4), and both partition exclusively into the detergent phase following extraction and phase separation with Triton X-114 (7, 27). However, inconsistencies between the physical properties (4, 7, 27) and

amino acid sequence (11) of the 47-kDa protein prompted us to investigate the possibility of fatty acid attachment to the molecule. In a companion report (3), it is shown that the 47-kDa antigen is a proteolipid. This finding was surprising, because recent DNA sequence analysis (11) predicted that the protein lacks a readily identifiable consensus cleavage site for signal peptidase II (39, 40) common to virtually all other procaryotic proteolipids (39, 40).

In this study, *E. coli* and *T. pallidum* both appeared to acylate the 47-kDa antigen; the apparent biochemical similarities between the native and recombinant proteolipids enabled us to examine the contribution of the covalently attached lipid to the amphiphilicity of the molecule. Our results suggest that the hydrophobicity of the 47-kDa antigen is dependent on the covalent addition of one or more fatty acids and that formation of the mature proteolipid does not require proteolytic protein processing. The ability of *E. coli* to acylate the 47-kDa antigen will facilitate determination of the exact linkage(s) between fatty acid(s) and the protein, as well as the influence of the lipid moieties on the extraordinary immunogenicity of this protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. *T. pallidum* subspecies *pallidum* (*T. pallidum*) (Nichols strain) was passaged continuously in New Zealand White male rabbits and extracted from testicular tissue as previously described (21, 35). *E. coli* RR1 (21) was used as a recipient of pGP1-2-pNC83, pGP1-2-pT7-4, and pNC81 (4, 20). Plasmids pGP1-2 and pT7-4 (36) were provided by Stanley Tabor and Charles Richardson (Harvard Medical School, Boston, Mass.). Plasmid pNC83 was made by first digesting pNC82 (4, 11) to completion with *Xba*I followed by partial digestion with *Hind*III. The products were electrophoresed in a preparative 1% agarose gel, and the fragment containing the 47-kDa gene was excised

* Corresponding author.

and electroeluted. This was ligated to pT7-4, previously digested to completion with *Xba*I and *Hind*III, and the ligation mixture was used to transform *E. coli* RR1 containing pGP1-2. The minicell-producing strain *E. coli* ORN103 (1, 9, 24) was the recipient of pUC19 and pPH47-2 (4, 11, 41).

Immunologic reagents. Murine monoclonal antibody 11E3, directed against the 47-kDa immunogen of *T. pallidum*, has been described previously (12, 16, 17). Monoclonal antibody 5C11 (Immunoglobulin G2b), directed against the 34-kDa antigen of *T. pallidum* (35), was generated by immunizing BALB/c mice with Triton X-114 extracts of *T. pallidum* (28). Rabbit antiserum reactive with the *T. pallidum* integral membrane proteins, designated as detergent-phase proteins, was prepared as described previously (27). Horseradish peroxidase-protein A conjugate was purchased from Sigma Chemical Co. (St. Louis, Mo.).

SDS-PAGE, Western blotting (immunoblotting), and 2DE. Samples to be analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were boiled for at least 3 min in final sample buffer (62.5 mM Tris hydrochloride, 10% glycerol, 2% SDS) and electrophoresed through a 4% stacking gel and an either 10 or 12.5% resolving gel (13). The gels were fixed and dried for autoradiography, enhanced for fluorography by saturation with 1 M salicylic acid (2), or transferred to nitrocellulose (0.2- μ m pore size; Schleicher & Schuell, Inc., Keene, N.H.) for Western blotting. Western blots were incubated with the appropriate monoclonal antibody and then with horseradish peroxidase-protein A conjugate and developed with 3 mg of 4-chloro-1-naphthol per ml and 0.03% hydrogen peroxide in 20 mM Tris hydrochloride (pH 7.6)–0.1 M NaCl. Two-dimensional gel electrophoresis (2DE) was performed as described by O'Farrell (23), with modifications described by Norris et al. (22); the second dimension consisted of 4% stacking and 10% resolving gels.

Extrinsic 125 I labeling and purification of the recombinant 47-kDa antigen. *E. coli* RR1 containing pGP1-2–pNC83 was grown overnight in 1 liter of L broth at 37°C, and cell envelopes were prepared as previously described (4). Cell envelopes in phosphate-buffered saline (PBS) were mixed with an equal volume of chloroform-methanol (2:1 [vol/vol]). The material at the interface was removed, suspended in 2 $\times$ final sample buffer, and electrophoresed through an SDS–10% PAGE gel. The region of the gel extending 5 mm above and below a 45-kDa molecular mass marker was excised, minced, and extracted overnight at 37°C in 10 volumes of PBS containing 0.05% SDS. The resulting extract was concentrated by using a Minicon macrosolute concentrator (molecular weight exclusion, 30,000; Amicon Corp., Lexington, Mass.). One-half of the concentrated extract was then added to Iodobeads (Pierce Chemical Co., Rockford, Ill.) preloaded with 2.0 mCi of 125 I (Amersham) and allowed to react for 6 min at room temperature. The beads were removed, and the solution was diluted to 15 ml in 50 mM Tris hydrochloride (pH 8.0)–150 mM KCl–5 mM EDTA containing 1% *n*-octyl glucoside (Calbiochem-Behring, La Jolla, Calif.). The radiolabeled 47-kDa antigen was then purified to homogeneity in a batchwise fashion by using an immunoaffinity matrix consisting of monoclonal antibody 11E3 conjugated to Reacti-Gel 6X (Pierce) as previously described (4).

Intrinsic radiolabeling of proteins in *T. pallidum*. Proteins of *T. pallidum* were labeled with Trans [35 S]label (1,165 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) or [9,10(*n*)- 3 H]palmitate (54 Ci/mmol; Amersham) by using 2.5 mCi of each radioactive compound in 10 ml of the labeling medium described by Stamm and Bassford (33). Organisms

were incubated for 20 h (34°C) at a final concentration of 10⁹/ml with 100 μ g of cycloheximide per ml (33). Treponemes were extracted with Triton X-114, and the washed detergent phase was immunoprecipitated with monoclonal antibody 11E3 (35).

Intrinsic radiolabeling of *E. coli* proteins. *E. coli* RR1 containing plasmids pGP1-2 and pT7-4 or pGP1-2 and pNC83 were grown to an optical density of 0.5 at 590 nm in L broth at 37°C. The *E. coli* (10 ml) cells were then centrifuged at 27,000 $\times g$ for 10 min, washed once in 20 ml of M9 medium, and centrifuged as described above. The bacteria were then suspended in 10 ml of M9 medium, and 100 μ Ci each of [9,10(*n*)- 3 H]palmitate (54 Ci/mmol; Amersham) were added. The organisms were incubated for 2 h at 37°C, centrifuged, boiled for 3 min in 1 ml of 20 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–1% SDS (TES), and diluted in 9 ml of 20 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–150 mM NaCl–1% Triton X-100 (TENT). The suspension was centrifuged at 27,000 $\times g$ for 10 min at 4°C, followed by immunoprecipitation of the supernatant with monoclonal antibody 11E3 (35). Immunoprecipitates were placed in final sample buffer, boiled for 3 min, separated by SDS-PAGE, and detected by fluorography.

Intrinsic radiolabeling of the recombinant 47-kDa antigen by a cell-free translation system. Five micrograms of pNC81 plasmid and 167 μ Ci of [L- 35 S]methionine (>8,000 Ci/mmol; Amersham) per ml were added to an in vitro transcription-translation kit (Amersham). After 1 h of incubation at 37°C, followed by a 5-min chase with a 5 mM unlabeled amino acid mixture, the reaction mixture was solubilized with 2% Triton X-114, phase separated (see below), and analyzed by SDS-PAGE and fluorography.

Labeling of the recombinant 47-kDa antigen in this system by using 1 mCi of [9,10(*n*)- 3 H]palmitate (54 Ci/mmol; Amersham) was also attempted (as described above), except that the amino acid chase solution was added at the beginning of the 1-h incubation period.

Processing of the recombinant 47-kDa antigen in *E. coli*. Samples (50 ml) of L broth containing 100 μ g of ampicillin per ml and 100 μ g of kanamycin per ml were inoculated with *E. coli* containing either pGP1-2–pT7-4 or pGP1-2–pNC83 and incubated at 37°C until cell growth reached an optical density of 0.53 at 590 nm. Six-milliliter portions of each culture were pelleted by centrifugation at 27,000 $\times g$ for 10 min (4°C). The cultures were washed with 37 ml of cold M9 medium, pelleted as described above, and suspended in 12 ml of M9 medium containing the appropriate antibiotics plus 20 μ g of thiamine hydrochloride per ml and 0.01% of each amino acid (except methionine and cysteine). Suspensions were incubated at 37°C for 2 h. Cultures were then pulsed for 2 min at 37°C with 180 μ Ci of Trans [35 S]label and chased by addition of unlabeled methionine to a final concentration of 0.1%. Duplicate 1-ml portions of each culture were taken at 0, 2, 5, 15, 60, and 120 min and placed in 0.5 ml of ice-cold stop solution (0.5 mg of chloramphenicol per ml, 0.4 M sodium azide, and 0.02 M 2,4-dinitrophenol). The samples were pelleted at 12,000 $\times g$ for 10 min at 4°C, boiled for 3 min in 100 μ l of TES, and then diluted with 900 μ l of TENT. Boiled samples were then centrifuged at 12,000 $\times g$ for 10 min and immunoprecipitated with monoclonal antibody 11E3 (12, 35); portions containing approximately 10,000 cpm of radioactivity were taken from each sample for analysis by SDS-PAGE and fluorography.

Radiolabeling of minicells and protein processing. *E. coli* minicells transformed with either pUC19 or pPH47-2 were prepared by the method of Frazer and Curtiss (9) and stored

at -70°C in M9 medium containing 20% (vol/vol) glycerol. Frozen minicells were thawed on ice, collected by centrifugation, and preincubated in M9 medium for 10 min at 37°C to allow for the breakdown of endogenous, preformed mRNA. Minicells were collected, suspended in M9 medium containing $50\text{ }\mu\text{Ci}$ of Trans [^{35}S]label per ml and $20\text{ }\mu\text{g}$ of cycloserine per ml, and incubated for 30 min at 37°C .

Indirect inhibition of signal peptidase I by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) (29) was used to investigate processing of the 47-kDa antigen in minicells. Minicells were suspended in 0.1 ml of M9 medium containing 0, 25, 50, or $75\text{ }\mu\text{M}$ CCCP plus Trans [^{35}S]label ($50\text{ }\mu\text{Ci/ml}$). Mixtures were incubated for 30 min at 37°C . The cells were centrifuged, and the pellet was suspended in 0.1 ml of $2\times$ final sample buffer for SDS-PAGE and fluorography.

Phase partitioning with Triton X-114. Samples were suspended in ice-cold PBS containing 2% Triton X-114 for extraction as previously described (27).

Production of synthetic peptides and KLH-peptide conjugates. Two peptides 12 amino acids (CAAKRGVLSRVS) and 23 amino acids (CAAMFDAVSRATHGHGAFRQQFQ) long, one corresponding to a predicted carboxy-terminal region between two stop codons of the 47-kDa antigen sequence (11) and the other corresponding to the amino-terminal region (11), respectively, were synthesized in a 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.) and assayed for purity as previously described (18, 30, 37). The peptides were cross-linked to keyhole limpet hemocyanin (KLH; 86 mg/ml in 50% glycerol; Calbiochem-Behring) by using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS; Pierce). KLH (40 mg in 0.465 ml) was placed in 3.54 ml of PBS (pH 6.0). MBS (6 mg) dissolved in $200\text{ }\mu\text{l}$ of dimethyl sulfoxide (Pierce) was added with vigorous stirring; the mixture was incubated for 30 min at room temperature. KLH-MBS was separated from the unreacted MBS by using a Sephadex G-25 column (void volume, 4 ml; inclusion volume, 10.7 ml). KLH-MBS was collected in the void volume as a gray cloudy material. The peptides (10 mg) were then dissolved in a solution composed of 1 ml of PBS-5 μl of 5 N NaOH-1 ml of dimethyl sulfoxide. One-half of the KLH-MBS solution from the Sephadex G-25 column was added to each of the peptides under vigorous vortexing. The mixture was incubated with stirring for 1 h at room temperature. KLH-peptide conjugates were stored at -20°C .

Production of anti-peptide antibodies. Rabbits were immunized at four sites by subcutaneous injections of peptide-KLH conjugates ($300\text{ }\mu\text{g}$ per rabbit) in complete Freund adjuvant (1:1) on day 0 and given subcutaneous injections of peptide-KLH ($300\text{ }\mu\text{g}$ per rabbit) in incomplete Freund adjuvant (1:1) on days 21, 42, and 91. Animals were bled on days 28 and 105. Production of antibody to the 47-kDa antigen was assayed by immunoblotting 1:100 dilutions of serum against solubilized treponemal proteins separated by SDS-PAGE (equivalent of 10^8 *T. pallidum* cells per gel lane) and electroeluted to nitrocellulose. Immunoblots were then reacted with HRP-protein A conjugate (1:1,000 of a 1-mg/ml stock solution; Sigma) to visualize the proteins.

RESULTS

Identities of native and recombinant DNA-derived 47-kDa immunogens. It was essential to confirm that the native and recombinant DNA-derived 47-kDa molecules were similar before *E. coli* recombinants could be used to investigate the biochemical properties of the treponemal protein. Affinity-

FIG. 1. 2DE analysis of native and recombinant DNA-derived 47-kDa immunogens. Unlabeled *T. pallidum* proteins and recombinant ^{125}I -labeled 47-kDa antigen were electrophoresed simultaneously, and the proteins were transferred to nitrocellulose, immunoblotted with monoclonal antibody, and autoradiographed. (A) Western blot of the 47-kDa antigen from *T. pallidum*. (B) Autoradiograph of the Western blot shown in panel A. The pH gradient used for isoelectric focusing (IEF) was from 7 to 5. Apparent molecular masses are in kilodaltons. SDS refers to the direction of the second dimension.

purified, ^{125}I -labeled recombinant 47-kDa antigen from *E. coli* was added to unlabeled, solubilized antigens of whole *T. pallidum*, and the mixture was simultaneously analyzed by 2DE. The unlabeled native 47-kDa antigen exhibited its previously described (4, 22) unusual mobility upon 2DE, as assessed by immunoblotting with monoclonal antibody; most of the antigen migrated with an acidic pI (approximately 5.5), while a portion also remained at the basic region of the gel (Fig. 1A). Autoradiography showed that the purified, radiolabeled recombinant DNA-derived antigen (synthesized by *E. coli*) (Fig. 1B) migrated with a 2DE mobility similar to that of the native molecule (Fig. 1A).

FIG. 2. Radioimmuno-precipitation of ^3H -labeled fatty acid-labeled 47-kDa antigen from *T. pallidum* or *E. coli*. Lanes: 1, radioimmuno-precipitation of *T. pallidum* with an irrelevant monoclonal antibody; 2, radioimmuno-precipitation of *T. pallidum* with specific monoclonal antibody 11E3; 3, radioimmuno-precipitation of *E. coli* lacking the 47-kDa antigen gene by monoclonal antibody 11E3; 4, radioimmuno-precipitation of *E. coli* containing the 47-kDa antigen gene (pGP1-2-pNC83) by using monoclonal antibody 11E3. The apparent molecular mass is in kilodaltons.

[^3H]palmitate labeling of the 47-kDa antigen in *T. pallidum* and *E. coli*. Previous studies using Triton X-114 phase partitioning have established the amphiphilicity of the native and recombinant 47-kDa proteins (4, 7, 27). In another study (3), we found that the 47-kDa antigen of *T. pallidum* is a proteolipid. [^3H]palmitate-labeled 47-kDa antigen was selectively immunoprecipitated by monoclonal antibody 11E3 from treponemes after extraction and phase separation with Triton X-114 (Fig. 2, lane 2). The identity of the native and recombinant molecules shown by 2DE in Fig. 1 suggested that the cloned 47-kDa antigen was similarly acylated in *E. coli*. To test this, *E. coli* with or without plasmids pGP1-2 and pNC83 was labeled with [^3H]palmitate, [^3H]oleate, and [^3H]myristate in combination to maximize the probability of labeling. The radiolabeled 47-kDa gene product was immunoprecipitable from *E. coli* containing pGP1-2-pNC83 (Fig. 2, lane 4) but not by the products of the cloning host alone (Fig. 2, lane 3). *E. coli* minicells containing another 47-kDa antigen-expressing construct, pPH47-2, also produced a ^3H -labeled fatty acid-labeled 47-kDa antigen immunoprecipitable with monoclonal antibody (data not shown).

Amphiphilicity of the 47-kDa antigen produced in *E. coli* minicells but not as produced in an *E. coli* cell-free system. To determine the contribution of covalently attached lipid to the amphiphilicity of the 47-kDa protein, phase partitioning with Triton X-114 was used to assess the relative hydrophobicities of the polypeptides produced from the 47-kDa antigen gene by either an in vitro or an in vivo protein translation system. Following synthesis, samples were extracted with Triton X-114 (phase partitioned into detergent and aqueous phases), and each phase was immunoprecipitated with either monoclonal or polyclonal antibodies by using protein A-bearing *Staphylococcus aureus* (Cowan I). In the cell-free system, an irrelevant monoclonal antibody and *S. aureus* immunoprecipitated products nonspecifically primarily from the detergent phase (Fig. 3A, lane 1). When monoclonal or polyclonal antibodies specific for the 47-kDa antigen were used in immunoprecipitations, a 47-kDa primary gene translation product was revealed in the aqueous phases (Fig. 3A,

FIG. 3. Triton X-114 detergent phase partitioning of the 47-kDa antigen expressed by an *E. coli* cell-free translation system or by *E. coli* minicells. The 47-kDa antigen synthesized either in vitro or in minicells was extracted with Triton X-114 and phase partitioned. Proteins from both the aqueous and detergent phases were radioimmuno-precipitated, separated by SDS-PAGE, and detected by autoradiography. (A) Radioimmuno-precipitation of proteins expressed by plasmid pNC81 in the cell-free translation system. The detergent (lane 1) and aqueous (lane 2) phases were immunoprecipitated by monoclonal antibody 5C11 (irrelevant monoclonal antibody). The detergent (lane 3) and aqueous (lane 4) phases were also immunoprecipitated by monoclonal antibody 11E3. Lanes 5 and 6, detergent and aqueous phases immunoprecipitated by rabbit antiserum to the detergent phase proteins (inclusive of the 47-kDa antigen) of *T. pallidum*, respectively. (B) Radioimmuno-precipitation of proteins expressed by plasmid pPH47-2 (a derivative of pNC81) in *E. coli* minicells and found in the detergent (lane 1) and aqueous (lane 2) phases of Triton X-114 by using monoclonal antibody 11E3. Apparent molecular masses are in kilodaltons.

lanes 4 and 6), along with some previously reported (4, 11) lower- M_r species of the 47-kDa antigen (lane 6). It was assumed that the heavily labeled product nonspecifically immunoprecipitated from all of the detergent phases represented the *tet* gene product, an integral cytoplasmic membrane protein encoded by the same plasmid vector; as expected, this product partitioned exclusively into the Triton X-114 detergent phase (Fig. 3A, lanes 1, 3, and 5). An entirely different result was obtained with the antigen produced by *E. coli* minicells; the 47-kDa antigen partitioned into and was immunoprecipitated predominantly from the detergent phase (Fig. 3B, lane 1). When the cell-free translation system was used to label the 47-kDa antigen with [^3H]palmitate, no radiolabeled product could be detected (data not shown).

Comparison of the primary 47-kDa gene translation product in *E. coli* with the native *T. pallidum* 47-kDa antigen. Processing by signal peptidase II is a feature common to other well-characterized bacterial proteolipids (39, 40). Recent amino acid sequence data for the 47-kDa antigen (11) did not reveal consensus cleavage sites for either signal peptidases I or II. However, complicating the interpretation of sequence data was the fact that the N terminus of the mature molecule could not be determined directly (11). Furthermore, a less abundant protein with an apparent molecular mass of 48 kDa is commonly observed along with the predominant 47-kDa antigen on immunoblots of either *T. pallidum* or *E. coli* recombinant clones by using polyclonal and monoclonal antibodies directed against this antigen (4, 11, 20, 22); it has

FIG. 4. Synthesis of the 47-kDa primary gene translation product by an *E. coli* cell-free translation system. Unlabeled *T. pallidum* antigens and the [35 S]methionine-labeled 47-kDa antigen from an *E. coli* cell-free translation system (containing pNC81) were placed in the same well of an SDS-PAGE gel, electrophoresed, and electroeluted onto nitrocellulose. The strip was autoradiographed (lane 2) and then Western blotted with monoclonal antibody 11E3 (lane 3). Lane 1, autoradiograph of [35 S]-labeled proteins translated from negative control plasmid pBR322 showing both processed and unprocessed β -lactamase. Apparent molecular masses are in kilodaltons.

been suggested that the 48-kDa component represents a precursor of the predominant species. Therefore, it was important to test directly whether the 47-kDa antigen is synthesized in a precursor form.

First, we compared the apparent molecular mass of the primary *in vitro* translation product of the 47-kDa antigen synthesized in a cell-free system with that of the native protein from *T. pallidum*. Monoclonal antibody immunoblotting of *T. pallidum* antigens revealed the 47- and 48-kDa doublet migration pattern characteristic of this protein (Fig. 4, lane 3) (4, 11, 22). The product of the 47-kDa antigen gene synthesized in a cell-free system comigrated with the smaller of the two native polypeptides (Fig. 4, lane 2), even though production of the doublet by intact *E. coli* recombinant clones has been well documented (4, 11, 20). An apparently higher-molecular-mass protein was not detected, even on prolonged overexposure of the fluorogram (data not shown). The presence of signal peptidase I in the *in vitro* transcription-translation system was suggested by the apparent partial processing (two species) of β -lactamase from the control plasmid (Fig. 4, lane 1).

Lack of *in vivo* processing of the recombinant 47-kDa antigen. Two different *in vivo* processing experiments were performed to confirm that the 47-kDa protein lacks a cleavable signal peptide. *E. coli* RR1 transformants containing pGP1-2-pNC83 were pulse-labeled with [35 S]methionine and chased for various times with unlabeled methionine. Fluorographs of immunoprecipitated material revealed a single

FIG. 5. Processing of the 47-kDa antigen in *E. coli* and *E. coli* minicells. (A) Row 1, autoradiograph of the 47-kDa antigen synthesized by *E. coli*(pGP1-2-pNC83) while pulsed with [35 S]methionine, followed by a chase with unlabeled methionine. Samples were removed at 0, 2, 5, 15, 60, and 120 min and subjected to immunoprecipitation with monoclonal antibody 11E3, SDS-PAGE, and autoradiography. Row 2, autoradiograph of β -lactamase from *E. coli* (pGP1-2-pT7-4), used as a positive control for the time-dependent processing of pre- β -lactamase (pre- β Lt) to mature β -lactamase (β Lt). *E. coli* containing pGP1-2-pT7-4 was pretreated with rifampin to inhibit host cell RNA polymerase but not T7 RNA polymerase, so that only plasmid-encoded β -lactamase would be expressed. (B) Row 1, autoradiograph of the 47-kDa antigen from *E. coli*(pPH47-2) minicells preincubated with 0, 25, 50, or 75 μ M CCCP. Row 2, β -lactamase from *E. coli*(pUC19) minicells treated with CCCP and analyzed as in row 1.

protein with an apparent molecular mass of 47 kDa at all sampling times (Fig. 5A, row 1). β -Lactamase, in contrast, was correctly processed under essentially identical conditions (Fig. 5A, row 2).

Furthermore, when *E. coli* minicells were incubated with [35 S]methionine and various concentrations of CCCP, a compound which prevents translocation of nascent polypeptides across the cytoplasmic membrane (29), a precursor for the 47-kDa antigen was not detected (Fig. 5B, row 1) while processing of β -lactamase was inhibited (Fig. 5B, row 2 [50 and 75 μ M]). Identical results were obtained by using ethanol inhibition of protein processing in *E. coli* minicells (25; data not shown).

Because it is not clear how closely the kinetics of protein processing in *T. pallidum* correspond to events that occur in *E. coli*, the results shown in Fig. 5 did not entirely exclude the possibility that the apparently higher-molecular-mass species of the native 47- and 48-kDa doublet represents an inefficiently processed precursor (8) not detected in *E. coli* processing experiments. Analysis of the amino acid sequence of the protein revealed a possible alternative explanation for the genesis of the 48-kDa component. The C terminus of the protein contains two stop codons separated by nine amino acids (11); occasional readthrough of the first stop codon would produce an additional species with a

FIG. 6. Western blots of *T. pallidum* antigens with anti-peptide sera. Lanes: 1, immunoblot using antiserum to the polypeptide spanning the two stop codons at the C terminus of the 47-kDa antigen; 2, immunoblot using antiserum against the 23-amino-acid polypeptide of the N terminus of the 47-kDa antigen. Apparent molecular masses are in kilodaltons.

molecular mass of approximately 48 kDa. As predicted, anti-peptide serum to the putative N terminus reacted with both the 48- and 47-kDa antigens of *T. pallidum* (Fig. 6, lane 2). In contrast, antiserum to the peptide predicted to extend between the two stop codons reacted only with the 48-kDa antigen (Fig. 6, lane 1).

DISCUSSION

This study was undertaken to investigate the basis for the biochemical properties of the major integral membrane immunogen of *T. pallidum*. Molecular analysis of this protein is ultimately important in elucidating its structural and functional role(s) in *T. pallidum*. The findings that both the native (*T. pallidum*) and recombinant DNA-derived (*E. coli*) forms of the antigen are hydrophobic, that they exhibit similar 2DE migration patterns, and that they possess similar, if not identical, proteolipid structures allowed us to pursue a genetic strategy using *E. coli* for analysis of the protein. It is noteworthy that a similar analysis could not have been performed with the noncultivable *T. pallidum*.

Previous studies have shown that the 47-kDa antigen is amphiphilic (4, 7, 27), although hydrophobic domains could not be readily identified in the primary sequence (11). This discrepancy encouraged us to examine alternative explanations for the amphiphilic character of the protein. Results described elsewhere (3) show that the native 47-kDa antigen is a proteolipid. In this study, we demonstrated that the recombinant antigen is similarly acylated and that lipid moieties contribute significantly to the hydrophobic properties of the antigen. In contrast to the 47-kDa antigen purified from *T. pallidum* (3), definitive biochemical evidence of covalent attachment of the lipid was not obtained for the

recombinant antigen because of its relatively poor expression in *E. coli* (4, 11). However, several lines of evidence support such analogous covalent attachment. (i) The antigen can be intrinsically labeled with ^3H -labeled fatty acids in either *E. coli* or *E. coli* minicells under conditions that have been demonstrated previously to label proteolipids in *E. coli* selectively (10). (ii) Radioactivity remained associated with the recombinant protein after boiling in SDS and subsequent resolution by SDS-PAGE.

Demonstration that the recombinant protein is acylated enabled us to determine the contribution of the lipid to the hydrophobicity of the molecule. It was hypothesized that a cell-free translation system may lack the enzymatic activity (or activities) necessary for acylation of the protein and that phase partitioning with Triton X-114 could be used to assess the hydrophobic character of the molecule after synthesis by either whole cells or cell extracts. This particular approach was used because the relatively minor molecular weight contribution of the lipid to the protein may not be expected to cause a readily discernible shift in SDS-PAGE mobility. A similar strategy was used by Pillai and Baltimore (26) to assess the role of the lipid in the membrane-bound form of immunoglobulin synthesized by a cultured line of pre-B cells. One of the important findings of our study was that, in contrast to the antigen synthesized by both *T. pallidum* and *E. coli*, the 47-kDa antigen translated in vitro did not display the hydrophobicity characteristic of this antigen (4, 7, 27). The finding that radiolabeling with ^3H -labeled fatty acids did not occur in the in vitro translation system supports the contention that the hydrophobicity of the 47-kDa antigen is conferred by the covalently attached fatty acid(s). However, these data do not preclude the possibility that unidentified portions of the polypeptide also function as membrane-anchoring domains. This is because rare integral membrane proteins that do not possess covalently bound lipid have been described which segregate anomalously into the aqueous phase during Triton X-114 phase partitioning (15), because protein modeling algorithms do not invariably correctly predict membrane-associated domains (e.g., porins [19]), and because the primary sequence of the 47-kDa protein does contain limited domains with a hydrophobic character (11). Nevertheless, with respect to a number of proteolipids, there is ample evidence that covalent lipid addition is a prerequisite for membrane association (31).

All of the well-characterized proteolipids of procaryotes contain a specific type of leader sequence (39, 40). However, the amino acid sequence of the 47-kDa antigen did not reveal a plausible signal peptide of any known type. Because the amino terminus of the mature protein is blocked to Edman degradation (11), the possibility remained that the start codon assigned on the basis of the DNA sequence was incorrect and that the antigen is, in fact, synthesized as a higher-molecular-mass precursor. Resolution of this point could be accomplished only by comparing the native 47-kDa antigen with the primary gene translation product and by conducting processing experiments with *E. coli*. The combined results provide convincing evidence that the 47-kDa antigen gene product does not undergo proteolytic processing.

A characteristic feature of the 47-kDa antigen produced either by *T. pallidum* or by *E. coli* recombinant clones and analyzed by SDS-PAGE is the frequent presence of a 48-kDa component that reacts with specific polyclonal and monoclonal antibodies (4, 11, 20, 22). It has been suggested that the 48- and 47-kDa doublet seen in this and other studies (4, 11, 20, 22) is the result of inefficient (8) and/or incomplete

processing of the 47-kDa antigen. Although it is considered unlikely, it was important to investigate this, particularly in light of the absence of demonstrable processing in *E. coli*. Immunoblotting with anti-peptide sera supported the previously proposed alternative explanation (11) for the presence of the 47- and 48-kDa doublet, namely, that occasional translational readthrough of an initial stop codon results in synthesis of the 48-kDa component in both *T. pallidum* and in *E. coli* (11).

On the basis of currently available data, it has not been possible to determine the nature and the sites of acylation of the 47-kDa protein. Although biochemical analyses using the 47-kDa antigen purified from *T. pallidum* are proceeding, the ability of *E. coli* to acylate the 47-kDa antigen may facilitate these structural analyses by providing larger quantities of higher-specific-activity labeled material for initial analysis. Identity between the native and recombinant molecules can then be confirmed by targeting the analogous native lipopeptide for examination. The expression of the 47-kDa antigen as a proteolipid in *E. coli* but as an unacylated form during cell-free translation may also allow us to examine the putative role of the lipid(s) in the immunogenicity of the protein.

ACKNOWLEDGMENTS

We thank Mary Brandt and Mark Swancutt for helpful discussions and reading the manuscript, Laura Andrews and Martin Goldberg for outstanding technical assistance, and Cindy Baselski for excellent typing.

This study was partially supported by grant V3-181-64 from the World Health Organization, grant I-940 from the Robert A. Welch Foundation, Public Health Service grants AI-16692 and AI-17366 from the National Institute of Allergy and Infectious Diseases to M.V.N., Public Health Service grant AI-26756 from the National Institute of Allergy and Infectious Diseases, and a grant from the Texas Advanced Technology Program to J.D.R. J.D.R. was a Pfizer new faculty scholar.

LITERATURE CITED

- Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree. 1967. Miniature *Escherichia coli* deficient in DNA. *Proc. Natl. Acad. Sci. USA* **57**:321-326.
- Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132-135.
- Chamberlain, N. R., M. E. Brandt, A. L. Erwin, J. D. Radolf, and M. V. Norgard. 1989. Major integral membrane protein immunogens of *Treponema pallidum* are proteolipids. *Infect. Immun.* **57**:2872-2877.
- Chamberlain, N. R., J. D. Radolf, P.-L. Hsu, S. Sell, and M. V. Norgard. 1988. Genetic and physicochemical characterization of the recombinant DNA-derived 47-kilodalton surface immunogen of *Treponema pallidum* subsp. *pallidum*. *Infect. Immun.* **56**:71-78.
- Chesney, A. M. 1926. Immunity in syphilis. *Medicine (Baltimore)* **5**:463-547.
- Clark, E. G., and N. Danbolt. 1964. The Oslo study of the natural course of untreated syphilis. An epidemiologic investigation based on a re-study of the Boeck-Bruusgaard material. *Med. Clin. N. Am.* **48**:613-623.
- Cunningham, T. M., E. M. Walker, J. N. Miller, and M. A. Lovett. 1988. Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. *J. Bacteriol.* **170**:5789-5796.
- Dallas, W. S., P. H. Ray, J. Leong, C. D. Benedict, L. V. Stamm, and P. J. Bassford, Jr. 1987. Identification and purification of a recombinant *Treponema pallidum* basic membrane protein antigen expressed in *Escherichia coli*. *Infect. Immun.* **55**:1106-1115.
- Frazer, A. C., and R. Curtiss III. 1975. Production, properties, and utility of bacterial minicells. *Curr. Top. Microbiol. Immunol.* **69**:1-84.
- Hayashi, S., and H. C. Wu. 1988. Identification and characterization of lipid-modified membrane proteins in bacteria, p. 88-93. In U. Brodbeck and C. Bordier (ed.), *Post-translational modification of proteins by lipids. A laboratory manual*. Springer-Verlag, New York.
- Hsu, P.-L., N. R. Chamberlain, K. Orth, C. R. Moomaw, L.-Q. Zhang, C. A. Slaughter, J. D. Radolf, S. Sell, and M. V. Norgard. 1989. Sequence analysis of the 47-kilodalton major integral membrane immunogen of *Treponema pallidum*. *Infect. Immun.* **57**:196-203.
- Jones, S. A., K. S. Marchitto, J. N. Miller, and M. V. Norgard. 1984. Monoclonal antibody with hemagglutination, immobilization, and neutralization activities defines an immunodominant, 47,000 mol wt, surface-exposed immunogen of *Treponema pallidum* (Nichols). *J. Exp. Med.* **160**:1404-1420.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Magnuson, H. J., E. W. Thomas, S. Olansky, B. I. Kaplan, L. De Mello, and J. C. Cutler. 1956. Inoculation syphilis in human volunteers. *Medicine (Baltimore)* **35**:33-82.
- Maher, P. A., and S. J. Singer. 1985. Anomalous interaction of the acetylcholine receptor protein with the nonionic detergent Triton X-114. *Proc. Natl. Acad. Sci. USA* **82**:958-962.
- Marchitto, K. S., S. A. Jones, R. F. Schell, P. L. Holmans, and M. V. Norgard. 1984. Monoclonal antibody analysis of the specific antigenic similarities among pathogenic *Treponema pallidum* subspecies. *Infect. Immun.* **45**:660-666.
- Marchitto, K. S., C. K. Selland-Grossling, and M. V. Norgard. 1986. Molecular specificities of monoclonal antibodies directed against virulent *Treponema pallidum*. *Infect. Immun.* **51**:168-176.
- Merrifield R. B. 1964. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85**:2149-2154.
- Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49**:1-32.
- Norgard, M. V., N. R. Chamberlain, M. A. Swancutt, and M. S. Goldberg. 1986. Cloning and expression of the major 47-kilodalton surface immunogen of *Treponema pallidum* in *Escherichia coli*. *Infect. Immun.* **54**:500-506.
- Norgard, M. V., and J. N. Miller. 1983. Cloning and expression of *Treponema pallidum* (Nichols) antigen genes in *Escherichia coli*. *Infect. Immun.* **42**:435-445.
- Norris, S. J., J. F. Alderete, N. H. Axelsen, M. J. Bailey, S. A. Baker-Zander, J. B. Baseman, P. J. Bassford, R. E. Baughn, A. Cockayne, P. A. Hanff, P. Hindersson, S. A. Larsen, M. A. Lovett, S. A. Lukehart, J. N. Miller, M. A. Moskopidis, F. Müller, M. V. Norgard, C. W. Penn, L. V. Stamm, J. D. van Embden, and K. Wicher. 1987. Identity of *Treponema pallidum* subsp. *pallidum* polypeptides: correlation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis results from different laboratories. *Electrophoresis* **8**:77-92.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
- Orndorff, P. E., and S. Falkow. 1984. Organization and expression of genes responsible for type 1 piliation in *Escherichia coli*. *J. Bacteriol.* **159**:736-744.
- Palva, E. T., T. R. Hirst, S. J. S. Hardy, J. Holmgren, and L. Randall. 1981. Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. *J. Bacteriol.* **146**:325-330.
- Pillai, S., and D. Baltimore. 1987. Myristoylation and the post-translational acquisition of hydrophobicity by the membrane immunoglobulin heavy-chain polypeptide in B lymphocytes. *Proc. Natl. Acad. Sci. USA* **84**:7654-7658.
- Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard. 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. *Infect. Immun.* **56**:490-498.
- Robertson, S. M., J. R. Kettman, J. N. Miller, and M. V. Norgard. 1982. Murine monoclonal antibodies specific for viru-

- lent *Treponema pallidum* (Nichols). Infect. Immun. **36**:1076–1085.
29. Russel, M., and P. Model. 1982. Filamentous phage pre-coat is an integral membrane protein: analysis by a new method of membrane preparation. Cell **28**:177–184.
30. Sarin, V. K., S. B. H. Kent, J. P. Tam, and R. B. Merrifield. 1981. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. Anal. Biochem. **117**:147–157.
31. Sefton, B. M., and J. E. Buss. 1987. The covalent modification of eukaryotic proteins with lipid. J. Cell Biol. **104**:1449–1453.
32. Sell, S., and S. J. Norris. 1983. The biology, pathology, and immunology of syphilis. Int. Rev. Exp. Pathol. **24**:203–276.
33. Stamm, L. V., and P. J. Bassford, Jr. 1985. Cellular and extracellular protein antigens of *Treponema pallidum* synthesized during in vitro incubation of freshly extracted organisms. Infect. Immun. **47**:799–807.
34. Stamm, L. V., W. S. Dallas, P. H. Ray, and P. J. Bassford, Jr. 1988. Identification, cloning, and purification of protein antigens of *Treponema pallidum*. Rev. Infect. Dis. **10**:S403–S407.
35. Swancutt, M. A., D. A. Twehous, and M. V. Norgard. 1986. Monoclonal antibody selection and analysis of a recombinant DNA-derived surface immunogen of *Treponema pallidum* expressed in *Escherichia coli*. Infect. Immun. **52**:110–119.
36. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
37. Tam, J. 1987. Synthesis of biologically active transforming growth factor alpha. Int. J. Pept. Protein Res. **29**:421–431.
38. Turner, T. B., and D. H. Hollander. 1957. Biology of the treponematoses. WHO Monogr. Ser. **35**:1–278.
39. Wu, H. C. 1987. Posttranslational modification and processing of membrane proteins in bacteria, p. 37–74. In M. Inouye (ed.), Bacterial outer membranes as model systems. John Wiley & Sons, Inc., New York.
40. Wu, H. C., and M. Tokunaga. 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immunol. **125**:127–157.
41. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.