

Protein D, the Immunoglobulin D-Binding Protein of *Haemophilus influenzae*, Is a Lipoprotein

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Protein D is an immunoglobulin D-binding membrane protein exposed on the surface of the gram-negative bacterium *Haemophilus influenzae*. Results reported here indicate that protein D is a lipoprotein. The protein is apparently synthesized as a precursor with an 18-residue-long signal sequence modified by the covalent attachment of both ester-linked and amide-linked palmitate to the cysteine residue, which becomes the amino terminus after cleavage of the signal sequence. Globomycin inhibited maturation of protein D in *H. influenzae*, implying that protein D is exported through the lipoprotein export pathway. A mutant expressing a protein D lacking the cysteine residue was constructed by oligonucleotide site-directed mutagenesis. The mutated protein D molecule was not acylated and partitioned in the aqueous phase after Triton X-114 extraction of intact bacteria, unlike native and recombinant protein D, which partitioned in the detergent phase. The nonacylated protein D molecule was localized to the periplasmic space of *Escherichia coli*. The hydrophilic protein D molecule will be used in investigations concerning its ability to function as a vaccine component.

Haemophilus influenzae type b is a major cause of meningitis and other invasive infections among children under the age of 4 in Europe and the United States (29, 31). Nontypeable *H. influenzae* is a common cause of otitis media and sinusitis in children (27). Patients with cystic fibrosis and chronic bronchitis often carry *H. influenzae*, which recently has been recognized as an important cause of pneumonia (27).

The current vaccines are based on purified type b capsular polysaccharide. The vaccines have proven to be effective against *H. influenzae* type b disease in children at the ages of 2 to 5 years. Children under the age of 2 respond poorly to the vaccine unless the polysaccharide is conjugated to protein carriers (12, 33-35). The polysaccharide vaccines do not protect against infections caused by nonencapsulated (nontypeable) *H. influenzae* strains, and therefore, surface-exposed proteins such as pili (6) and outer membrane proteins (7, 11, 14, 17) have been investigated in order to find a vaccine effective against both type b and nontypeable *H. influenzae*. Four *H. influenzae* outer membrane proteins have been shown to give rise to protective antibodies in immunized animals. These proteins include the P1 heat-modifiable outer membrane protein (21), the major P2 porin protein (26), the P6 peptidoglycan-associated lipoprotein (25), and a surface protein with an apparent molecular weight of 98,000 (17). A protein chosen as a vaccine component must be highly conserved in order to give protection from infection by heterologous strains. Of the vaccine candidates mentioned above, at least P2 has been shown not to protect against challenge by heterologous strains (26).

Recently, a 42-kDa membrane-associated immunoglobulin D (IgD)-binding protein, named protein D, was described (32). Protein D was found in all of the 127 *H. influenzae* strains tested for direct binding of IgD to their surfaces (1). By using four different monoclonal antibodies directed against protein D, it was also shown that the IgD-binding molecule is highly conserved (1). Cloning and sequencing of

the gene encoding protein D (*hpd*) revealed that protein D differs from all other characterized immunoglobulin-binding proteins in its basic structure (16). The deduced amino acid sequence of protein D suggested that protein D is a lipoprotein, since the signal sequence contains a consensus sequence for bacterial lipoproteins, Leu-Ala-Gly-Cys, as amino acids 16 to 19 (16, 40). In *H. influenzae*, at least 12 lipoproteins are found (39); one of these corresponds to protein D in size. When a lipoprotein is secreted, the amino-terminal cysteine residue is posttranslationally modified by the addition of a glycerol moiety containing ester-linked fatty acids. Then, signal peptidase II, a signal peptidase specific for lipoproteins, cleaves the signal peptide off, and the newly formed amino terminus is further acylated with an amide-linked fatty acid. The fatty acids linked to the amino-terminal cysteine residue then function as membrane anchors (for a review, see reference 40). To investigate whether protein D is a lipoprotein, bacteria were labeled with tritiated fatty acids. To evaluate if the hydrophobicity is determined by the acylation of the cysteine residue, a mutant lacking the cysteine was constructed. In this study, we report that protein D is a lipoprotein and that a mutated protein lacking the target site for acylation cannot be acylated and becomes hydrophilic.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and media. *Escherichia coli* JM83 (41) was used as recipient for plasmids pUC18 (41), pHIC348 (16) (carrying the *hpd* gene), and pHIC501 (this study). *E. coli* JM101 (41) and SDM (38) were used as hosts for M13mp18 bacteriophages (41). *E. coli* SDM was supplied with the T7-GEN in vitro mutagenesis kit (United States Biochemical, Cleveland, Ohio). *H. influenzae* 772 (32) was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with NAD and hemin (Sigma Chemical Co., St. Louis, Mo.), each at 10 µg/ml. *E. coli* strains were grown in Luria-Bertani (LB) broth (22) or 2× YT media (23). LB agar and 2× YT agar contained, in addition, 1.5 g of agar per liter. LB broth and

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LB agar were supplemented with ampicillin (Sigma) at 100 µg/ml for broth and 50 µg/ml for plates when bacteria carrying plasmids were cultured.

DNA isolations. Plasmid DNA and the replicative form of phage M13 were prepared by using a Qiagen plasmid DNA kit (Diagen GmbH, Düsseldorf, Germany) according to the instructions of the manufacturer. Single-stranded DNA from phage M13 clones was prepared from single plaques as previously described (23). DNA fragments were isolated from 1.0% agarose gels by extraction with a GeneClean kit (Bio 101, Inc., La Jolla, Calif.) as recommended by the supplier.

Restriction enzyme analysis. Restriction enzymes were purchased from Boehringer-Mannheim GmbH (Mannheim, Germany) and were used according to the instructions of the manufacturer.

Oligonucleotide site-directed mutagenesis. A 647-bp *Hind*III-*Eco*RI fragment of plasmid pHIC348 (16) was ligated into phage M13mp18 replicative-form DNA digested with the same enzymes. Competent *E. coli* JM101 was transformed (24) with the ligation mixture and plated onto 2× YT supplemented with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside and IPTG (isopropyl-β-D-thiogalactopyranoside). Single-stranded DNA was prepared from recombinant phages.

An oligonucleotide, 5'-CTAGCAGGTGGTAGCAGCCA-3', complementary to the region of *hpd* encoding the consensus sequence for signal peptidase II, was synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden). The oligonucleotide was phosphorylated before being used in mutagenesis experiments by using the procedure described in the protocols included in the T7-GEN in vitro mutagenesis kit purchased from United States Biochemical. Mutations were confirmed by DNA sequence analysis on single-stranded DNA as previously described (16).

SDS-PAGE, Western blot (immunoblot) analysis, and autoradiography. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a modified Laemmli gel (19). All samples that contained Triton X-114 were acetone precipitated before they were suspended in sample buffer (0.06 M Tris hydrochloride [pH 6.8], 2% [wt/vol] SDS, 1% [vol/vol] β-mercaptoethanol, 10% glycerol, 0.03% [wt/vol] bromophenol blue) and boiled for 5 min prior to loading. Delipidated [³H]palmitate-labeled proteins were suspended in sample buffer and boiled for 5 min prior to loading. After electrophoresis, gels were either stained with Coomassie brilliant blue or electroblotted (37) to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). For identification of protein D, membranes were incubated either with 20 µg of human myeloma IgD 4490 per ml (32) or with monoclonal antibody 16C10 (1) for 2 h at 23°C. Membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-human IgD immunoglobulins (Sigma) at a dilution of 1:1,000 or with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts a/s, Glostrup, Denmark) at a dilution of 1:2,000 and developed as previously described (16). Gels that contained molecules labeled with [³H]palmitate were treated with En³Hance (DuPont, NEN Research Products, Boston, Mass.) before being dried and fluorographed according to the directions of the manufacturer. The treated gels were exposed to X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) at -70°C for 3 to 14 days with intensifying screens.

Extraction of *H. influenzae* and *E. coli* with Triton X-114. *H. influenzae* 772, *E. coli* JM83(pHIC348), and *E. coli* JM83(pHIC501) were extracted with Triton X-114 essen-

tially according to the method described by Swancutt et al. (36). Briefly, 100-ml cultures of bacteria were grown to an optical density at 550 nm of 0.5 (approximately 3 × 10⁸ CFU/ml), harvested by centrifugation at 4,000 × *g* for 10 min at 4°C, and washed with 1 volume of 200 mM Tris HCl (pH 8.0). The cell pellets were suspended in 1 ml of 20 mM Tris HCl (pH 8.0)-10 mM EDTA-2% Triton X-114. After incubation for 4 h at 4°C, cellular debris was removed by centrifugation. The supernatants were removed and warmed to 37°C in a water bath to allow phase separation to occur. After centrifugation for 10 min at 13,000 × *g*, the upper aqueous phase was separated from the detergent phase. The aqueous phases were cleansed by addition of 10% Triton X-114 to a final concentration of 2%, and the detergent phases were diluted to the original volume by adding 20 mM Tris HCl (pH 8.0)-10 mM EDTA at 0°C (30). The washing procedure was repeated three times.

Cell fractionation. Osmotic shock treatment of *E. coli* carrying either pHIC348 or pHIC501 was carried out essentially as described by Heppel (15). Cells were harvested from 40-ml mid-logarithmic-phase cultures in LB supplemented with ampicillin, washed twice in 10 mM Tris hydrochloride (pH 7.3)-30 mM NaCl, and suspended in 33 mM Tris hydrochloride (pH 7.3)-0.1 mM EDTA-0.5 M sucrose (80:1 [vol/wt]). The cells were left at 23°C for 10 min prior to centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the cells were suspended in ice-cold 0.5 mM MgCl₂ (80:1 [vol/wt]). The samples were aliquotted into two portions of identical volumes. One aliquot was centrifuged as described above, while the other was kept on ice until sonicated. The supernatant containing periplasmic proteins was collected. The pellet was washed once in 10 mM Tris hydrochloride (pH 8.0) before suspension in the original volume of MgCl₂. Supernatants, pellets, and whole-cell extracts were sonicated to lyse any remaining cells. Membranes and debris were pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C. The supernatants containing periplasmic, cytoplasmic, and cytoplasmic-periplasmic extracts were collected. The membrane pellets were washed once in 10 mM Tris hydrochloride (pH 8.0) before being suspended in sample buffer.

The amount of cell lysis in the periplasmic extracts was estimated by comparing the malate dehydrogenase (MDH) activity in the periplasmic fractions with the activity in the cytoplasmic fractions and the supernatants of sonicated cells. The latter sample was taken to represent 100% lysis. MDH activity was determined as the oxaloacetate-dependent oxidation of NADH (42). The degree of release of periplasmic proteins was assayed by measuring the β-lactamase activities in the different fractions with nitrocefin (Oxoid Ltd, Basingstoke, United Kingdom) as a substrate. Samples (1 to 10 µl) of each fraction were incubated with 10 mg of nitrocefin in a total volume of 1 ml of 50 mM potassium phosphate (pH 7.0)-1 mM EDTA at 30°C. The increase in A₄₈₆ was determined immediately after addition of sample. The β-lactamase activities in the supernatants of sonicated intact cells were taken to represent 100% of the activity.

Intrinsic radiolabeling of bacterial proteins with [³H]palmitate. *H. influenzae* 772 was labeled with [9,10(*n*)-³H]palmitic acid (53.4 Ci/mmol; Amersham International, Amersham, England) according to the procedure of Weinberg et al. (39). The labeled cells were harvested by centrifugation and washed with phosphate-buffered saline. The bacterial pellet was suspended in 20 mM Tris HCl (pH 8.0)-1 mM EDTA-1% SDS and boiled for 5 min. Insoluble residue was removed by two rounds of centrifugation at 13,000 × *g* for 10

min at 4°C. *E. coli* JM83(pHIC348), JM83(pHIC501), and JM83(pUC18) were labeled by the same protocol.

In experiments in which the antibiotic globomycin was used to study protein processing, globomycin solubilized in ethanol was added to a final concentration of 150 µg/ml 10 min prior to the addition of [³H]palmitic acid. Final ethanol concentration was 0.2%. Control experiments without any addition of globomycin but with the addition of 0.2% ethanol were run parallel with the globomycin experiments.

Delipidation of [³H]palmitate-labeled proteins. Proteins were precipitated from lysates or Triton X-114 extracts by the addition of 10 volumes of ice-cold acetone (36). Lipids that were not covalently attached to proteins were removed by repeated chloroform-methanol extractions (3) until no further radioactivity was released.

Hydrolysis of [³H]palmitate-labeled protein D. Aqueous-phase and detergent-phase proteins prepared by Triton X-114 extraction of [³H]palmitate-labeled *H. influenzae* and *E. coli* cells were precipitated with 10 volumes of acetone. Each pellet was delipidated as described above. The pellets were suspended and boiled in sample buffer, and the proteins were separated by SDS-PAGE. After being stained with Coomassie brilliant blue and destained, protein bands corresponding to protein D were excised from the gel. A protein band corresponding to a nonradiolabeled protein was also excised as a control for background radioactivity. Sequential alkaline and acid hydrolyses were performed after three 2-ml washes with 50% methanol (5). Released fatty acids were extracted according to the method described by Bligh and Dyer (3).

Amino acid sequence analysis. Automated amino acid sequence analysis was performed on mutated protein D released by osmotic shock (15, 16) from *E. coli* JM83 harboring pHIC501. The released proteins, generally considered to be of periplasmic origin, were loaded onto a SDS-polyacrylamide gel and electroblotted to Immobilon membrane as described above. Strips were stained with Ponceau S (Sigma), and a band corresponding to protein D was excised from unstained membrane. The amino acid sequence analysis was performed as described previously (32).

RESULTS

Site-directed mutagenesis of *hpd*. In order to investigate the functional role of the cysteine 19 residue in protein D as a site for acylation, a mutant in which this amino acid was substituted for a glycine was constructed by site-directed mutagenesis in phage M13 as outlined in Fig. 1. The 675-bp *HindIII-EcoRI* fragment in pHIC348 was cloned into phage M13mp18 before being subjected to the mutagenesis reaction directed by the oligonucleotide, which carried a single T-to-G mutation. Six putatively mutated plaques were chosen for DNA sequencing. All six clones carried the shift from T to G at the correct position (results not shown). Apart from this single substitution, the clones were identical to the wild-type pHIC348. Replicative-form DNA was prepared from one of the mutants, and a 359-bp *HindIII-NheI* fragment carrying the mutation was isolated. This fragment was then ligated with pHIC348 DNA lacking the corresponding wild-type *HindIII-NheI* fragment to create the plasmid pHIC501. The gene carrying the cysteine 19-to-glycine 19 substitution is designated *hpd-1*.

Triton X-114 extraction of bacteria. It has previously been reported that protein D was localized to the membrane even though a hydropathy plot (18) indicated that the protein was hydrophilic (16). Hydrophobic proteins such as integral

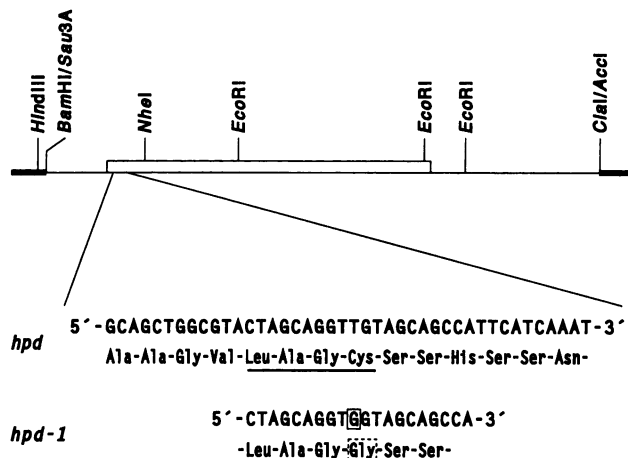


FIG. 1. Partial restriction enzyme map of *hpd* and outline for site-directed mutagenesis. Only restriction enzyme sites employed in subcloning for mutagenesis are indicated. Vector DNA is indicated by a thick line. Nucleotides 34 to 75 and amino acids -7 to +7 of *hpd* are shown. The consensus sequence for signal peptidase II is underlined. The single T-to-G substitution in the oligonucleotide is boxed, and the resulting amino acid substitution is marked with a dashed box.

membrane proteins partition in the detergent phase after Triton X-114 extraction (4). It was therefore interesting to compare the partitioning of the mutated protein D molecule (protein Dm) lacking the putative membrane anchor with native *H. influenzae*- and *E. coli*-expressed protein D after Triton X-114 extraction. Intact *H. influenzae* 772, *E. coli* JM83(pHIC348), and *E. coli* JM83(pHIC501) cells were extracted with Triton X-114. Insoluble and soluble materials were separated, and the soluble material was then partitioned into an aqueous and a detergent phase. Most proteins were insoluble in Triton X-114 or remained present in the aqueous phase (Fig. 2A). Only a limited number of proteins were present in the detergent phase (Fig. 2A). The solubilized protein D from *H. influenzae* 772 was exclusively found in the detergent phase (Fig. 2B, lane 3). Protein D expressed from pHIC348 partitioned mostly to the detergent phase. Less than 10% as estimated from the stained blot was found in the aqueous phase (Fig. 2B). Protein Dm expressed from pHIC501 exclusively partitioned in the aqueous phase (Fig. 2B, lane 8).

Localization of protein Dm. An experiment in which *E. coli* cells carrying pHIC348 or pHIC501 were subjected to osmotic shock (15) was performed in order to investigate the localization of protein Dm (Fig. 3). The mutated form of protein D was found mainly in the periplasmic fraction, whereas nonmutated protein D was found in the membrane fraction. Weak bands were observed in the cytoplasmic fractions (Fig. 3). The degree of cell lysis was monitored by measuring the leakage of the cytoplasmic enzyme MDH into the periplasmic fractions. The efficiency of release of periplasmic proteins was assayed by measuring the activity of the periplasmic enzyme β -lactamase. Less than 10% of the total MDH activity was found in the periplasmic fractions, and less than 3% of the total β -lactamase activity was found in the cytoplasmic fractions (Table 1). Protein Dm encoded by pHIC501 appeared to migrate slightly faster in SDS-PAGE than pHIC348-encoded protein D (Fig. 2B and 3).

Labeling of protein D with [³H]palmitate. To determine whether protein D is a lipoprotein, bacteria expressing the

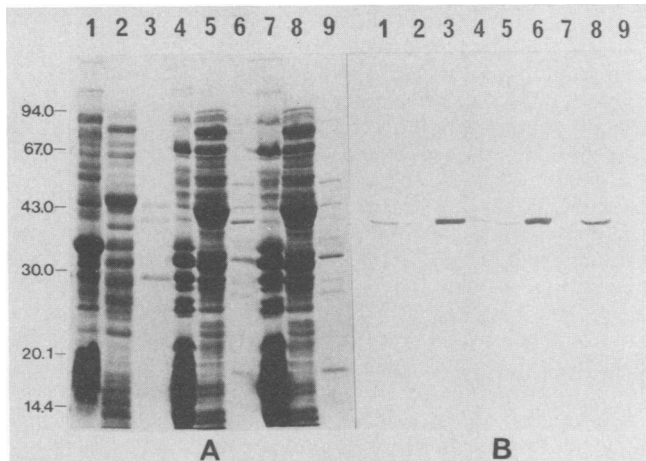


FIG. 2. Triton X-114 extraction and phase partitioning of proteins from *H. influenzae* 772, *E. coli* JM83(pHIC348), and *E. coli* JM83(pHIC501). Solubilized proteins were acetone precipitated prior to SDS-PAGE through 10% T-3.3% C polyacrylamide gels. Triton X-114-insoluble residue and precipitated proteins were suspended in sample buffer and boiled for 5 min under reducing conditions prior to loading. (A) Coomassie brilliant blue staining; (B) Western blot with human myeloma IgD. Lanes 1, Triton X-114-insoluble proteins of *H. influenzae* 772; lanes 2, aqueous fraction of *H. influenzae* 772; lanes 3 detergent fraction of *H. influenzae* 772; lanes 4 to 6, insoluble, aqueous, and detergent fractions of *E. coli* JM83(pHIC348), respectively; lanes 7 to 9, insoluble, aqueous, and detergent fractions of *E. coli* JM83(pHIC501), respectively. Numbers on the left give molecular sizes in kilodaltons.

protein were labeled with [³H]palmitic acid. Delipidated extracts were analyzed by SDS-PAGE and subjected to fluorography (Fig. 4). A protein in the cell extracts from *H. influenzae* 772 and *E. coli* JM83(pHIC348) with electrophoretic mobility identical to that of protein D was found to contain covalently bound radiolabeled fatty acid (Fig. 4,

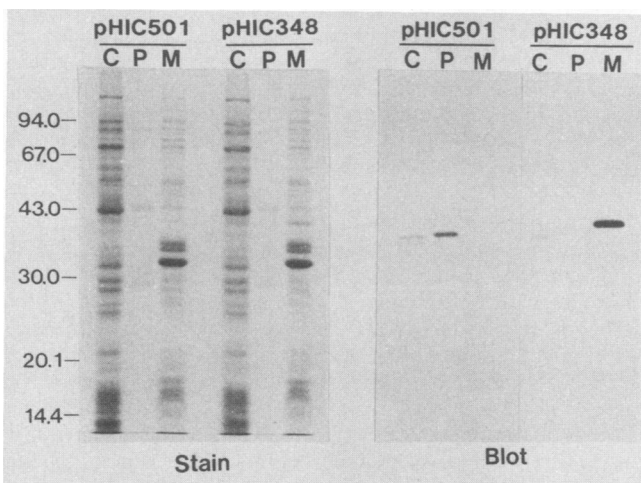


FIG. 3. Fractionation of protein D expressed from *E. coli* JM83(pHIC501) and *E. coli* JM83(pHIC348) analyzed by SDS-PAGE (10% T, 3.3% C). Left panel, Coomassie brilliant blue stain; right panel, Western blot using monoclonal antibody 16C10. Lanes C, cytoplasmic fraction; lanes P, periplasmic fraction; lanes M, membrane fraction. Equivalent volumes of extracts were loaded on the gels. Numbers on the left give molecular sizes in kilodaltons.

TABLE 1. Enzyme activities in protein extracts

Fraction	% Activity	
	MDH ^a	β-Lactamase ^b
pHIC501 periplasmic	<9	78
pHIC501 cytoplasmic	60	<3
pHIC348 periplasmic	<9	85
pHIC348 cytoplasmic	72	<3

^a 100% activity = 250 nmol of NADH oxidized per min per ml of protein extract.

^b 100% activity = 780 nmol of nitrocefin hydrolyzed per min per ml of protein extract.

lanes 1 and 2). In *E. coli* JM83(pHIC501) and *E. coli* JM83(pUC18), the corresponding bands were absent (Fig. 4, lanes 3 and 4). Western blot analysis of palmitate-labeled proteins showed that protein Dm could be detected in the *E. coli* JM83(pHIC501) lysate (results not shown). From these experiments, it is concluded that protein D is acylated in *E. coli* and that the cysteine residue at position 19 is involved in acylation of the *E. coli*-expressed protein D molecule. In the *H. influenzae* extract, it is possible that a protein of the same electrophoretic mobility as protein D is acylated. However, in SDS-PAGE analysis, when *H. influenzae* cells were labeled with [³H]palmitic acid in the presence of the antibiotic globomycin, which is known to specifically inhibit the processing of lipoproteins, the IgD-binding band and the radiolabeled 42-kDa band were found in slightly higher positions than protein from bacteria grown in the absence of globomycin (Fig. 5).

Analysis of ³H-fatty acid linkage to protein D. To verify that the ³H radiolabeling of protein D was due to the covalent attachment of radioactive fatty acids, a band corresponding to protein D was excised from SDS-PAGE gels of Triton X-114 phase extracts of [³H]palmitate-labeled *H. influenzae* 772 and *E. coli* JM83(pHIC348). The gel slices were subjected to sequential alkaline and acid hydrolyses. Approximately 80% of the radioactivity was released after the

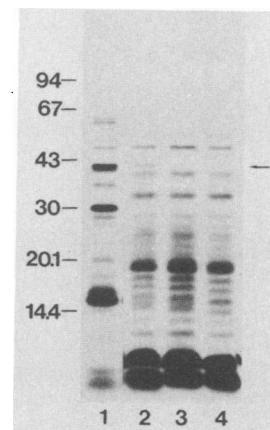


FIG. 4. Lipoproteins of *H. influenzae* and *E. coli* from whole-cell lysates of [³H]palmitic acid-labeled cells. Approximately 150,000 cpm was loaded in each well and submitted to SDS-PAGE through an SDS-12.5% T-3.3% C polyacrylamide gel. The stained and destained gel was treated with En³Hance before being dried and fluorographed for 8 days at -70°C. Lane 1, *H. influenzae* 772; lane 2, *E. coli* JM83(pHIC348); lane 3, *E. coli* JM83(pHIC501); lane 4, *E. coli* JM83(pUC18). The position of protein D is indicated by an arrow. Numbers on the left are molecular sizes in kilodaltons.

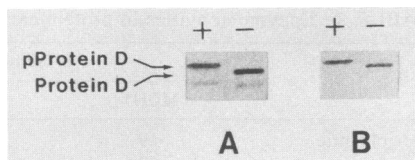


FIG. 5. Globomycin inhibition of lipoproteins from *H. influenzae* 772. The proteins were analyzed on an SDS-10% T-3.3% C polyacrylamide gel. The fluorograph (A) and immunoblot (B) were done with human myeloma IgD of whole-cell lysates prepared from [3 H]palmitate-labeled bacteria grown in the presence (+) or absence (-) of globomycin. The Immobilon membrane was treated with En 3 Hance prior to fluorography. Only that part of the blot displaying protein D is shown. The positions of proprotein D (pProtein D) and native protein D (Protein D) are given.

alkaline hydrolysis, whereas 20% was released after subsequent hydrolysis in acid, showing that protein D contains both ester-linked (sensitive to NaOH) and amide-linked (sensitive to acid) fatty acids.

Determination of N-terminal amino acid sequence of protein Dm. Attempts to determine the N terminus of protein D from *H. influenzae* have previously been unsuccessful (32). A compilation of N termini from other bacterial lipoproteins clearly shows that it is the acylated cysteine residue that forms the N terminus after removal of the signal sequence (40). From the [3 H]palmitate labeling experiments, we knew that mutated protein D was not acylated. To verify that the site of acylation, i.e., the cysteine residue, was removed in the mutated form of protein D expressed from pHIC501, amino acid sequencing was carried out on protein D excised from an Immobilon membrane. The amino-terminal sequence is shown in Fig. 6 in comparison with the expected sequence of processed native protein D.

DISCUSSION

This study was undertaken to investigate the basis for the biochemical properties of the IgD-binding protein of *H. influenzae*. Several lines of evidence suggest that protein D is a lipoprotein. The amino-terminal sequence of the *hpd* gene product show strong similarities to those of precursors of known bacterial lipoproteins (40). Protein D contains covalently attached fatty acids, and processing of protein D is inhibited by globomycin in *H. influenzae* 772. Furthermore, a mutated form of protein D that lacks the site of acylation could not be acylated and became hydrophilic. Amino-terminal sequencing showed that the *hpd-1* gene product was processed at a different site than native protein D. The increased electrophoretic mobility of the *hpd-1* gene product in SDS-PAGE (Fig. 2 and 3) might be explained by the lack of five amino acid residues and covalently attached fatty acids. Even though five amino acid residues are missing in the amino-terminal end of protein Dm compared with native protein D, it is unlikely that these amino acids are involved in anchoring the protein to the membrane, since

	19		33
Protein D	*C S S H S S N M A N T Q M L S		
Protein Dm		S N M A N T Q M L S	

FIG. 6. Amino-terminal sequence of protein Dm in comparison with predicted amino-terminal sequence of native protein D. Site of acylation is denoted with an asterisk. Numbers represent amino acid positions beginning from the translational start (16).

they are polar or positively charged. The finding that protein Dm could not be labeled with 3 H-labeled fatty acids supports the contention that the hydrophobicity of protein D is conferred by the covalently attached fatty acid(s). Even though there are strong indications that the acylation of protein D determines its hydrophobicity, the possibility that the membrane association is also due to other factors cannot be excluded. Protein-modeling algorithms do not invariably correctly predict membrane-associated domains, since some proteins that appear hydrophilic in hydropathy plots are membrane associated because of their folding patterns and aggregation (e.g., porins [28]). Our results do not exclude the possibility that it is conformational changes that alter the behavior of protein Dm compared with protein D. The physical properties of protein Dm must be compared with the properties of protein D to clarify whether the folding influences hydrophobicity in our experiments.

The glycerylcysteine lipoproteins generally are thought to contain 2 mol of ester-linked fatty acid per 1 mol of amide-linked fatty acid (40). Our data obtained from exogenous incorporation of [3 H]palmitate into protein D suggest a 4:1 ratio of ester-linked to amide-linked fatty acids. However, our data are based on the distribution of 3 H-fatty acids. We have not demonstrated that the precursor pools for ester- and amide-linked fatty acids are identical. Thus, we can conclude from our data that protein D contains both ester- and amide-linked fatty acids, but chemical data will be needed to determine the stoichiometry of ester-linked to amide-linked fatty acids.

Protein D might be a strong candidate for inclusion in a vaccine directed against *H. influenzae*. The protein D molecule is highly conserved among different strains (1). It is exposed on the surface of the bacteria, since there is a binding of radiolabeled IgD to intact bacteria (1, 16). However, one potential factor limiting the usefulness of protein D is its lipoprotein nature, which makes the protein hard to purify. The expression of the *hpd* gene in *E. coli* from its endogenous promoter is relatively weak, and if the insert is inverted to facilitate a better expression, the tandem arrangement of the *lacZ* promoter of the vector and the *hpd* promoter is toxic to the bacteria (16). Nonacylated protein D encoded by pHIC501 is hydrophilic (Fig. 2). Preliminary data show that this protein can be purified by ion-exchange chromatography from periplasmic extracts of *E. coli* harboring pHIC501. The purified nonacylated protein D could then be used in investigations in which the immunological response to protein D is studied. The non-lipid-containing protein D molecule may not have the same immunological properties as the native protein, since the lipid attached to lipoproteins has been proven to be mitogenic to lymphocytes (2). However, in experiments with a nonacylated form of *H. influenzae* lipoprotein PAL (P6), it was demonstrated that the nonacylated protein (rPAL) was as immunogenic as the native protein and that rPAL gave rise to protective antibodies in animal models (13).

The IgD-binding upper respiratory tract pathogens *H. influenzae* and *Moraxella (Branhamella) catarrhalis* (9) stimulate B lymphocytes to proliferation. For *M. catarrhalis*, this proliferation is due to interactions with surface IgD and class I major histocompatibility complex antigens on the surface of the B lymphocytes (10). Similar experiments with *H. influenzae* have not yet been published, but preliminary experiments indicate that the mitogenic response of human lymphocytes to *H. influenzae* is similar to that to *M. catarrhalis*. Injection into mice of goat anti-mouse IgD stimulates a large IgG1 anti-goat IgG antibody response as well as

polyclonal IgG1 production (8). Experiments in which antigen was conjugated to anti-IgD antibody gave up to 1,000-times-higher antibody titers than those in which antigen was injected alone (20), indicating that interaction with IgD expressed on the surface of lymphocytes is a very important step in B- and T-cell activation. Further investigations will be performed in order to investigate whether protein D can activate lymphocytes in the way antibodies directed against surface IgD do and give rise to large antibody responses in individuals immunized with protein D.

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