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Putative *Treponema pallidum* Cytadhesins Share a Common Functional Domain

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Three putative *Treponema pallidum* ligands (P1, P2, and P3) that bind host fibronectin were characterized by peptide mapping. Papain digestion of each protein yielded a comigrating peptide of approximately 12,000 molecular weight. An antibody to this protein fragment inhibited *T. pallidum* host cytadherence, indicating that this peptide may be the functional domain of these treponemal adhesins.

Adherence of *Treponema pallidum* to host cells appears to be an early event in the pathogenesis of syphilis (2, 4, 5, 7, 8). Our strategy was to characterize the treponemal and host cell surfaces for ligand and receptor identification as well as to understand better the specificity and nature of the overall parasite-host cell interaction. Three distinct outer envelope treponemal proteins (P1 [89,500], P2 [37,000], and P3 [32,000]; molecular weight values corrected from those given in previous publications [2, 9]) were previously characterized and implicated as adhesins (2, 5). No precursor-product or disulfide-linked relationships were evident among these treponemal proteins (unpublished data), although an antigenic relatedness between two of the three putative ligands was reported (2).

Additionally, the ligand candidates were found to interact with cell-surface fibronectin (9). Recent studies in our laboratory demonstrate the specificity of the *T. pallidum*-fibronectin association (10). Saturable, high-affinity *T. pallidum* acquisition of the cell-binding domain of fibronectin provided evidence for the existence of a single class of treponemal receptor-binding proteins that interact with fibronectin.

The current investigation was undertaken to characterize further the three putative ligands. Specifically, we were interested in determining whether each adhesin molecule contained an identical structural-functional domain relative to fibronectin recognition. In this report we show by immunobiochemical techniques the relatedness of these three treponemal ligand proteins. Additionally, we implicate a polypeptide common to each ligand as the biologically important domain involved in host cell parasitism by *T. pallidum*.

T. pallidum (Nichols) was harvested from infected rabbit testicles as described previously (1). Treponemes were washed in phosphate-buffered saline, and total treponemal proteins were precipitated in 10% trichloroacetic acid. For some experiments, freshly extracted organisms were radiolabeled with [^{35}S]methionine (specific activity, 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or Na^{125}I (Amersham) (1, 2) prior to trichloroacetic acid precipitation of proteins.

Trichloroacetic acid-precipitated treponemal proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 14-cm-long slab gels, 7.5% acrylamide). A strip of gel containing electrophoresed molecular weight standards and total *T. pallidum* proteins was stained with Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo.). Protein bands were excised and processed immediately or frozen at −20°C. Gel bands were macerated, and treponemal proteins were electroeluted from the acrylamide gel by using a model 1750 electrophoretic concentrator (ISCO, Lincoln, Nebr.). Electroeluted ligand proteins P1, P2, and P3 possessed mobilities equal to those of the corresponding protein in the original preparation (Fig. 1A). Peptide mapping was performed as described by Cleveland et al. (6) by using Hoefer 4-cm-long minigels and 10 or 15% polyacrylamide gels. Proteolytic digestion of each ligand was conducted with chymotrypsin, trypsin, *Staphylococcus aureus* V8 protease, and papain (all from Sigma) in microcentrifuge tubes at 37°C for 60 min. Digestion was terminated by addition of 0.25 M 2-mercaptoethanol and 2% SDS and boiling for 3 min before SDS-PAGE on 15% acrylamide gels. Electrophoresis was conducted at 15 and 30 mA in the stacking and separating gels, respectively. Controlled proteolysis was also performed by equilibration of specific protein-containing gel pieces for 30 min in 0.125 M Tris buffer, pH 6.8, before enzyme digestion in the stacking gel (6). Results were similar to those generated with electroeluted proteins.

Antiserum to proteins P2 and P3 was prepared by immunization of rabbits with acrylamide protein preparations. Briefly, stained bands corresponding to the P2 and P3 regions of acrylamide gels were excised and lyophilized after preparative SDS-PAGE of trichloroacetic acid-precipitated treponemal proteins from 2×10^{10} spirochetes. The gel pieces were pulverized and mixed with complete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.) for subcutaneous and intramuscular injections into rabbits. Boosters given at days 10 and 20 were in incomplete Freund adjuvant. Sera were collected at days 30 and 60 and examined for antibody by radioimmunoprecipitation assays (2, 3). Anti-P2-P3 serum possessed an antibody to proteins limited to the P2-P3 molecular weight region (Fig. 1B). In contrast, rabbit syphilitic serum (RSS) contained antibodies to most or all treponemal proteins (2).

After proteolytic digestion and electrophoresis, peptides were blotted onto nitrocellulose by a modification of the method of Towbin et al. (11). Transfer was performed at 0.2 A for 16 h. Transfer buffer contained 0.15 M glycine, 0.02 M Tris, and 20% methanol, pH 8.3. Blots were then probed with RSS (2, 4) or rabbit anti-P2-P3 serum (Fig. 1B). After extensive washing, blots were incubated with ^{125}I-protein A as recently described (10, 11).

Immunoblots of electroeluted P1, P2, and P3 digested with

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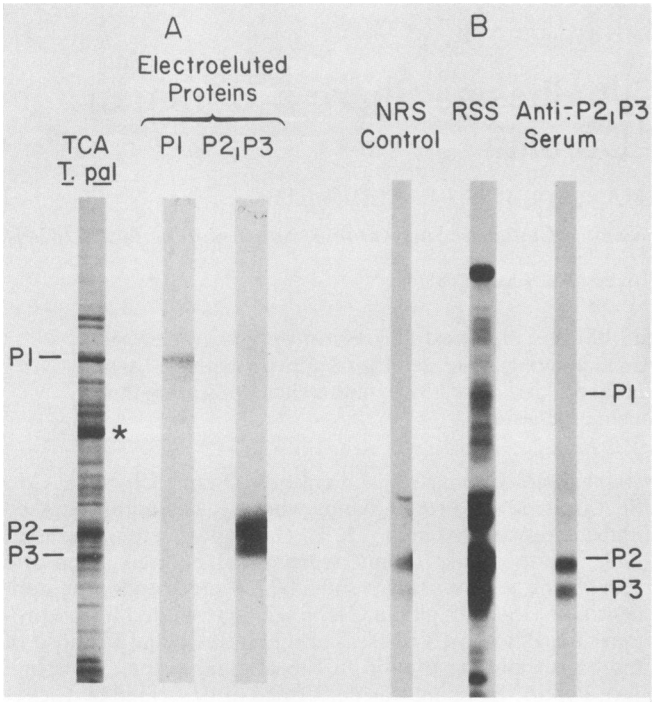

FIG. 1. Coomassie brilliant blue-stained *T. pallidum* protein bands after SDS-PAGE (A) and fluorograms of ^{35}S-treponemal proteins after radioimmunoprecipitation assay (B). (A) Patterns of *T. pallidum* proteins are compared with those of stained bands of electroeluted proteins after electrophoresis on 12-cm-long gels of 7.5% acrylamide. Implicated ligands are designated as P1, P2, and P3 (left margin). Asterisk refers to P5, identified previously as another outer envelope protein (2), which was used as a control for immunoblot experiments (Table 1). TCA, Trichloroacetic acid. (B) Individual proteins were immunoprecipitated from a detergent extract of ^{35}S-labeled *T. pallidum* incubated with normal rabbit serum (NRS), RSS, and anti-P2-P3 serum. SDS-PAGE was conducted on 15-cm-long gels of 7.5% acrylamide before fluorography.

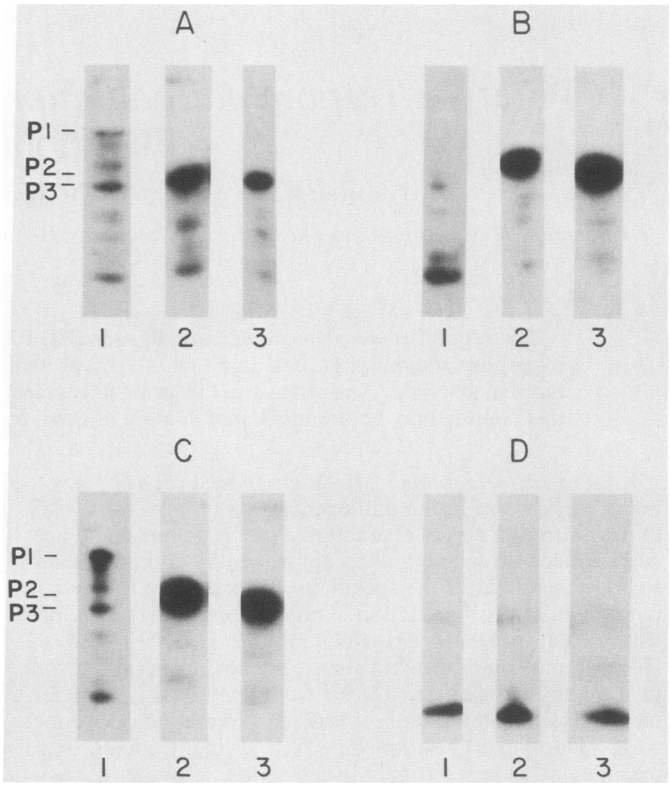

FIG. 2. Immunoblots of *T. pallidum* protein digests after SDS-PAGE on 4-cm-long minigels of 15% acrylamide. Treponemal proteins were digested with 5 μg each of trypsin (A), chymotrypsin (B), *S. aureus* V8 protease (C), and papain (D). Lanes 1, 2, and 3 in each section represent proteins P1, P2, and P3, respectively. Electrophoretic migrations of undigested P1, P2, and P3 are indicated.

four different enzymes are shown in Fig. 2. Greater P1 sensitivity to trypsin, chymotrypsin, and *Staphylococcus aureus* V8 protease was noted when compared with proteins P2 and P3. In contrast, papain readily digested the three treponemal proteins to a comigrating peptide. It is noteworthy that the resulting protease-digested peptide fragment appeared equal in intensity to the original, undigested protein band. The same results were obtained when proteins from intrinsically and extrinsically labeled treponemal proteins were processed similarly. Additionally, similar digest patterns resulted when peptide mapping was performed with proteins P1, P2, and P3 affinity-purified on fibronectin-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) (9). No treponemal proteins were detected in immunoblot experiments performed with normal rabbit serum as control.

Papain was then used for exhaustive digestion of proteins P1 and pooled P2 and P3 in an attempt to identify the smallest common proteolytic fragment. In each digestion, immunoblotting with RSS detected only one low-molecular-weight protein band (Fig. 3B) when compared with duplicate samples of undigested proteins (Fig. 3A). Anti-P2-P3 serum reacted with the digested treponemal proteins in a manner similar to that of RSS (Fig. 3C). Five determinations yielded an average molecular weight of 12,000 for this comigrating peptide (Fig. 3B). These data appear to show an antigenic relatedness among the putative ligands, reinforcing previous reports (2, 9) on the structure-function homologies between these proteins.

Antibody to the common domain (Fig. 3C) was then eluted in 100 mM glycine, pH 2.2, with 20 mM magnesium acetate

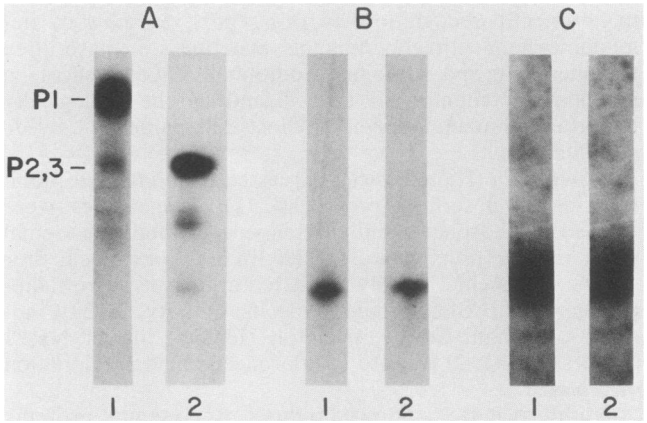

FIG. 3. Immunoblot detection of P1, pooled P2 and P3, and papain-digested peptide after electrophoresis on 4-cm-long gels of 15% acrylamide. (A) Immunoblots of undigested P1 (lane 1) and P2 and P3 (lane 2). (B and C) Immunoblots of digested P1 (lane 1) and P2 and P3 (lane 2) after incubation with 5 μg of papain for 60 min at 37°C and reaction of nitrocellulose with RSS (B) or anti-P2-P3 serum (C). Locations of the blots of proteins P1, P2, and P3 are indicated.

TABLE 1. Representative inhibition of *T. pallidum* cytadherence to HEp-2 monolayers by antibody to a protease-digested peptide common to treponemal proteins P1, P2, and P3[a]

Treatment reagent[b]	Concn (μg/ml)	Mean recovered cpm ± SD[c]	% of control
Medium control		26,455 ± 3,218	100
IgG to P1 papain digest peptide	20	20,673 ± 2,663	78.1
	40	15,467 ± 2,098	58.5
IgG to P2, P3 papain digest peptide	20	15,135 ± 2,817	57.2
	40	13,892 ± 2,760	52.5
IgG to P1, P2, and P3 papain digest peptide (pooled)	20	16,721 ± 1,984	63.1
	40	15,103 ± 2,116	57.0
IgG to P5[d]	20	25,716 ± 2,433	97.2
	40	24,933 ± 2,602	94.2

[a] P1, P2, and P3 are described in the legend to Fig. 1. Procedures for measuring attachment of *T. pallidum* to cell monolayers are as recently detailed (9,10). For this experiment ^{35}S-treponemes (7×10^7) were incubated in a 1-ml total volume with Dulbecco minimal essential medium or antibody for 30 min at 34°C. The suspensions were added to HEp-2 monolayers in Leighton tubes. After incubation for 2 h at 34°C, the cover slips were removed, washed in phosphate-buffered saline, and counted for radioactivity.

[b] Antibody was eluted from nitrocellulose blots treated with RSS as described in the text, and IgG was purified by protein A-Sepharose chromatography. Purity was tested by SDS-PAGE before use in this study, and protein determinations were performed by using the Bradford reagent (Bio-Rad Laboratories, Richmond, Calif.).

[c] Three separate determinations.

[d] P5 is the designation for a treponemal outer envelope protein (Fig. 1) used as a control.

and 50 mM KCl and was purified on protein A-Sepharose. The immunoglobulin G (IgG) was then tested for possible inhibition of treponemal cytadherence to HEp-2 cell monolayers (9, 10). Table 1 shows that preincubation of *T. pallidum* with IgG to the P1 or P2-P3 common domain inhibited treponemal attachment to HEp-2 cell monolayers. No similar inhibition was observed with culture media, IgG from normal rabbit serum, or RSS IgG eluted from an immunoblot of a different treponemal outer envelope protein, P5 (Fig. 1) (2). These data indicate that the comigrating peptide common to P1, P2, and P3 may be the functional domain of the treponemal adhesins.

Although the immunoblot of undigested P1 (Fig. 3) gave minor bands comigrating with P2 and P3, the putative adhesins of *T. pallidum* appear distinct since no precursor-product or disulfide bonding among the proteins could be demonstrated (2, 5, 9). The comigrating minor bands (Fig. 3), therefore, may result from the extensive experimental manipulations of the proteins involved in these studies. This report does, however, indicate that these three proteins (P1, P2, and P3) possess a structurally related region. The existence of a common domain among these surface proteins is consistent with data showing a single class of treponemal binding proteins for fibronectin (10). Demonstration of a common protease-resistant fragment of the three key treponemal protein ligands (Fig. 2) may be meaningful in view of the critical nature of the cytadherence event to infection and disease pathogenesis (5, 7, 8). Furthermore, since antibody to this common domain inhibited treponemal adherence to host cells (Table 1) and to fibronectin-coated surfaces (data not shown), the usefulness of the common domain peptide as a vaccine candidate or a diagnostic reagent is of interest. Studies are in progress to ascertain the unique structure-function features of this shared peptide region in its association with the fibronectin molecule (10).

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