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Defining the Interaction of the *Treponema pallidum* Adhesin Tp0751 with Laminin

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Various invasive pathogens attach to host tissues via the extracellular matrix component laminin, the major glycoprotein found within basement membranes. Previous investigations identified the laminin-binding adhesin Tp0751 within the spirochete bacterium *Treponema pallidum*. In the current study, Tp0751 was shown to attach to a variety of laminin isoforms that are widely distributed throughout the host, including laminins 1, 2, 4, 8, and 10. Such universal attachment is conducive for an adhesin present within a highly invasive pathogen that encounters a variety of tissue sites during the course of infection. Additional studies systematically identified the amino acid residues within Tp0751 that contribute to laminin binding using synthetic peptides designed from the mature protein sequence. The minimum laminin-binding region of the adhesin was localized to 10 amino acids; peptides containing these residues inhibited attachment of Tp0751 and *T. pallidum* to laminin. Further, Tp0751-specific antibodies inhibited attachment of *T. pallidum* to laminin. This study furthers our knowledge of the interaction of *T. pallidum* with laminin, an association that is proposed to facilitate bacterial traversal of basement membranes and subsequent entry into the circulation and tissue invasion. As such, these investigations will reveal new targets for possible prevention of bacterial dissemination and establishment of chronic infection.

Syphilis is a multistage disease caused by infection with the spirochete bacterium *Treponema pallidum* subsp. *pallidum*. In 1995, the World Health Organization's global estimate of annual new syphilis cases was 12 million, with the majority of the cases occurring in developing nations (24). Within the last several years a rapid increase in the number of cases occurring in eastern Europe has been observed (1, 34), and recent outbreaks have been reported among men who have sex with men in cities across Europe and North America (2, 9, 49). Further, infectious syphilis directly impacts human health through two additional routes: congenital syphilis continues to be an important pediatric health concern worldwide (78), and syphilis infection leads to an increased risk of transmission and acquisition of the human immunodeficiency virus (53).

Despite community outreach programs, the rates of primary and secondary syphilis have been steadily increasing in the United States over recent years, with a 12.4% increase in reported cases observed between the years 2001 and 2002 and an overall syphilis rate of 2.4 cases per population of 100,000 (9). The limited effectiveness of both local and global public health programs to control syphilis emphasizes the need for implementation of alternative means of syphilis prevention and specifically highlights the need for a greater understanding of the pathogenic mechanisms used by *T. pallidum* to establish and sustain infection.

Treponema pallidum gains entry to the host through intact mucosal barriers or microscopic epidermal abrasions (57). The pathogen has limited toxigenic properties, and tissue destruction associated with the disease appears to be due to the strong inflammatory response mounted by the host following infection (64). The organism is highly invasive; treponemes disseminate widely within hours of infection in experimental animals (11, 59), and in vitro studies have shown that *T. pallidum* is able to penetrate intact membranes and endothelial cell monolayers (62, 69). Treponemal invasion results in widespread bacterial dissemination, which in turn sets the stage for establishment of chronic infection.

Little is known about the virulence mechanisms utilized by invasive pathogens. No universal proteins contributing to bacterial invasion and dissemination have been identified, and proteins involved in these processes generally exhibit little or no sequence conservation. Several proteins involved in adhesion, invasion, and/or dissemination have been characterized in pathogens that maintain an intracellular lifestyle at some point during the course of infection. Substantially less information is currently available about extracellular pathogens and the virulence factors facilitating initiation of infection and spread to distant tissue sites. One factor which appears to be shared among many invasive pathogens, both intracellular and extracellular, is the capacity to attach to extracellular matrix (ECM) components. In particular, interaction with ECM components has been associated with the invasive ability of various pathogens (54), and the ECM component laminin is specifically targeted by a number of disseminating pathogens, including *Paracoccidioides brasiliensis* (76), *Histoplasma capsulatum* (47), *Toxoplasma gondii* (23), virulent mycobacteria (56, 66), *Candida albicans* (27), *Sporothrix schenckii* (40), and *Leishmania donovani* (25). In addition, tumor cells specifically interact with laminin during metastasis and dissemination, and a recent study has indicated that a unique tumor cell line with increased metastatic potential shows enhanced laminin attachment (51).

Laminins are a growing family of large multidomain heterotrimeric glycoproteins that comprise the most abundant

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FIG. 1. Overview of the synthetic peptide sequences used to delineate the minimum laminin-binding sequence of Tp0751. Thirteen synthetic peptides were prepared to cover the Tp0751 mature protein sequence (p1 to p13). Also depicted are the scrambled peptides (p4-scr, p6-scr, and p10-scr) and the mutated peptides that were created, including the peptides with each of the four amino acids unique to peptides 4, 6, and 10 mutated, either collectively (m4, m6, and m10) or individually (98P-A through 185S-A). The predicted signal sequence for Tp0751 is shown by a double underline, and the four amino acids unique to each of peptides 4, 6, and 10 are identified by a single underline.

component of the basement membrane, a specialized dense sheet of ECM that separates the epithelium from the connective tissue and surrounds the vascular endothelium, muscle cells, lipocytes and nerve cells (10, 71, 72). *T. pallidum* must cross several basement membranes throughout the course of infection, such as during both intravasation and extravasation, and during invasion of other tissues, including muscles or nerves. Previous literature has documented the capacity of *T. pallidum* to attach to laminin-coated surfaces (22), and prior investigations have identified a *T. pallidum* laminin-binding adhesin, Tp0751, via bioinformatic analyses performed on the *T. pallidum* genome and subsequent ECM attachment assays (7). Tp0751 is expressed during infection and exhibits a strong affinity for laminin (7). In this report, we further investigate the laminin attachment capability of the Tp0751 adhesin in order to identify potential targets for inhibition of bacterial dissemination and prevention of chronic infection.

MATERIALS AND METHODS

***T. pallidum*.** *T. pallidum* subsp. *pallidum* (Nichols strain) was propagated in New Zealand White rabbits as described elsewhere (44). All animal studies were approved by the local institutional review boards and conducted in accordance with standard accepted principles.

Laminin sources. Laminins isolated from Engelbreth-Holm-Swarm (EHS) murine sarcoma (laminin 1) and human placenta (laminin 2/4) were purchased from Sigma (St. Louis, Mo.). Recombinant human laminin 8 and laminin 10 were a generous gift from Masayuki Doi (Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan).

Synthetic peptides. Peptides were synthesized by Bio-WORLD (Dublin, OH) using Fmoc (9-fluorenylmethoxycarbonyl)-based solid phase chemistry. Peptides were purified using high-performance liquid chromatography, and the purity and identity of the synthetic peptides were confirmed by high-performance liquid chromatography and mass spectroscopy, respectively. Peptides were reconstituted in dimethyl sulfoxide to a concentration of 80 mg/ml, followed by dilution to the appropriate working concentration with phosphate-buffered saline (PBS), pH 7.4. Thirteen overlapping peptides were synthesized that spanned the Tp0751 amino acid sequence from residue 46 through 237 (Fig. 1). This encompassed the entire mature Tp0751 sequence, including eight amino acids of the predicted signal sequence (7). Each peptide was 24 amino acids in length and shared 10 overlapping residues with the preceding and subsequent peptides. Scrambled peptides were prepared that used the exact amino acid composition of the original peptide but in a scrambled organization. Mutagenized peptides, corresponding to peptides 4, 6, and 10, were prepared that incorporated alanine or glycine residues in place of the four amino acids that are unique to each peptide (refer to Fig. 1). Except for the synthetic peptides used in the peptide inhibition experiment, all Tp0751 peptides incorporated six histidine residues at the amino terminus to allow for detection.

Recombinant protein expression and purification. His_6-tagged recombinant forms of Tp0751 produced included Tp0751-1 (encompassing amino acids 54 to 173) and Tp0751-2 (encompassing amino acids 98 to 199). The latter protein was expressed and used in select experiments involving the Tp0751-derived peptides, since this protein is inclusive of the amino acid residues determined to be involved in laminin attachment. The negative control recombinant protein Tp0557 was produced as described elsewhere (7). Expression of each of the recombinant proteins was performed using the pRSETc T7 expression vector and the *E. coli* expression strain BL21 Star (DE3) (both from Invitrogen,

Carlsbad, CA). Recombinant proteins were expressed, purified, and renatured as previously described (7, 8, 81). Quantitation of each of the recombinant proteins was performed with the BCA protein assay kit (Pierce, Rockford, IL).

Laminin-binding adherence assays. Laminin-binding adherence assays were performed as described previously (7). To determine the specificity of Tp0751 for various laminin isoforms, wells were coated with laminin isolated from EHS murine sarcoma (laminin 1) or human placenta (laminin 2/4) or with recombinant human laminin 8 or laminin 10. For all other laminin-binding adherence assays, wells were coated with EHS laminin. Wells were washed three times with PBS-0.05% Tween 20 (PBST) and blocked for 30 min with 1% bovine serum albumin (BSA). Recombinant Tp0751 proteins or synthetic Tp0751 peptides were incubated for 1.5 h at a concentration of 2 μg per well diluted in PBS. For the peptide inhibition assays, Tp0751-2 (2 μg per well) was added in the presence of non-histidine-tagged Tp0751 synthetic peptides p1, p4, p6, and p10, either individually or in combination, at concentrations ranging from 0 to 30 μM. Wells were washed six times with PBST, and adherent recombinant proteins and histidine-tagged synthetic peptides were detected via nickel-labeled horseradish peroxidase and the 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (both from Kirkegaard & Perry Laboratories, Gaithersburg, MD) as previously described (7). All incubations were performed at 37°C. Plates were read at 600 nm with an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Winooski, VT), and statistical analyses were performed with the Student two-tailed *t* test.

Cell adhesion assay. The colon carcinoma cell line SW480 was obtained from ATCC (Rockville, MD), and cells were maintained in Dulbecco's modified Eagle medium (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (HyClone). To determine the capacity of Tp0751 to mediate cell attachment, assays were performed as described previously (63). Briefly, 96-well non-tissue-culture-treated enzyme-linked immunosorbent assay plates (Fisher Scientific, Pittsburgh, PA) were coated for 24 h at 4°C with 100 μl of either the recombinant *T. pallidum* protein Tp0751-1, the negative control protein BSA, or the positive control protein fibronectin, all at a concentration of 20 μg/ml in PBS. Wells were blocked with 1% BSA prepared in serum-free Dulbecco's modified Eagle medium. Confluent SW480 cells were detached by treatment with 2 mM EDTA for 10 min at 37°C. Detached cells were collected by centrifugation for 5 min at 200 × *g* and washed once by centrifugation with serum-free medium. Fifty thousand cells were added to each of the wells, and plates were centrifuged for 1 min at 30 × *g* to ensure uniform settling of cells and incubated for 3 h at 37°C. Following the incubation, nonadherent cells were removed by centrifugation (top side down) at 30 × *g* for 1 min. The attached cells were fixed and stained with 1% formaldehyde, 0.5% crystal violet. After washing with PBS, adherence was determined by measuring absorption at 595 nm in a microplate reader (Bio-Rad, Hercules, CA). The data are reported as the mean absorbance of triplicate wells ± standard error of the mean (SEM).

Antiserum. Anti-Tp0751 and anti-Tp0155 (negative control) polyclonal sera were raised in New Zealand White rabbits by immunizing them five times at 3-week intervals with 125 μg of either Tp0751-1 or Tp0155 recombinant protein emulsified in the Ribi adjuvant system (Sigma). Normal rabbit serum was collected prior to the initial immunization, and immune rabbit serum was collected from rabbits infected with *T. pallidum* for >90 days.

***T. pallidum* inhibition assays.** For the treponemal adherence peptide inhibition assays, Lab-Tek II chamber slides (Nunc, Rochester, NY) were coated for 1.5 h at room temperature with 4 μg of laminin diluted in saline. The slides were washed once with saline and then incubated overnight at 4°C with either saline (negative control) or 250 μg of the following peptides, either individually or in combination: 1, 2, and 3; 4, 6 and 10; or m4, m6, and m10. Slides were washed three times with saline, followed by the addition of 1.5×10^8 treponemes per well. Slides were incubated for 3 h at 34°C under anaerobic conditions and washed gently with saline (eight times for 5 min each). For the treponemal adherence antibody inhibition assay, chamber slides were coated for 16 h at 4°C with 6 μg of laminin diluted in saline. Wells were washed two times with saline and blocked with 3% BSA-saline for 4 h at room temperature. During the blocking step, 4.5×10^7 treponemes were preincubated at 34°C under anaerobic conditions with a 1:2 dilution of either immune rabbit serum, normal rabbit serum, or anti-Tp0155- or anti-Tp0751-specific polyclonal serum. Control wells were incubated under the same conditions with no antibody addition. Following blocking, wells were washed two times with saline, and the treponeme-antibody mixtures or control treponeme sample were introduced and incubated for 2 h at 34°C under anaerobic conditions. After gentle washing with saline (six times for 5 min each), treponemes were fixed with 4% paraformaldehyde and washed an additional five times with saline. For each assay, attached spirochetes were visualized by dark-field microscopy and quantitative attachment was determined by calculating the number of attached treponemes per field. The assays were blinded, and a total of six fields were read for each attachment condition.

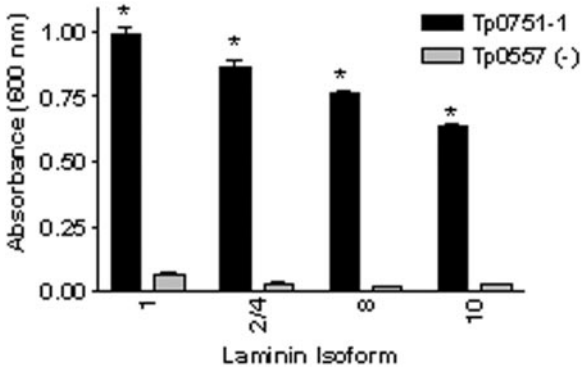

FIG. 2. Tp0751 Attachment to various laminin isoforms. The attachment potential of Tp0751-1 to laminin isoforms 1 (α1β1γ1), 2/4 (α2β1, 2γ2), 8 (α4β1γ1), and 10 (α5β1γ1) was determined. Each bar represents the mean absorbance value at 600 nm ± SEM for triplicate samples. For statistical analyses, attachment of Tp0751-1 to each of the laminin isoforms was compared with attachment of the negative control recombinant protein Tp0557 by the Student two-tailed *t* test (*, $P < 0.0001$).

RESULTS

Tp0751 attachment to various laminin isoforms. To determine the specificity of Tp0751 for different laminin isoforms, attachment assays were conducted using the laminin isoforms 1 (α1β1γ1), 2/4 (α2β1,2γ2), 8 (α4β1γ1), and 10 (α5β1γ1) and the Tp0751-1 recombinant preparation previously shown to mediate attachment to laminin (7). The results of these attachment studies are shown in Fig. 2. Recombinant Tp0751-1 demonstrated a significant level of attachment to each of the selected laminin isoforms ($P < 0.0001$), whereas minimal binding of the negative control recombinant protein Tp0557 to each of the laminin isoforms was observed.

Delineation of the Tp0751 amino acid residues involved in laminin attachment. Several experimental techniques were used to define the amino acid residues present within Tp0751 that are essential for attachment to laminin. In the first of these experiments, 13 synthetic 24-mer peptides covering the entire mature Tp0751 sequence were tested for their capacity to attach to laminin. As shown in Fig. 3A, peptides 4, 6, and 10 were the only peptides to exhibit attachment to laminin, thus suggesting that amino acid residues contained within these peptides mediate laminin attachment. Scrambled versions of peptides 4 and 6 did not attach to laminin, indicating that the interaction of each of these peptides with laminin is sequence dependent. A scrambled version of peptide 10 exhibited a similar level of attachment to laminin compared with that observed for the nonscrambled version of the peptide, suggesting that the amino acid composition of this peptide, and not the linear peptide sequence, is responsible for the observed laminin attachment potential of this segment of the protein. Attachment assays performed using a second scrambled version of peptide 10 showed a similar level of binding (data not shown).

To further investigate the peptide sequences involved in the interaction of Tp0751 with laminin, peptide inhibition studies

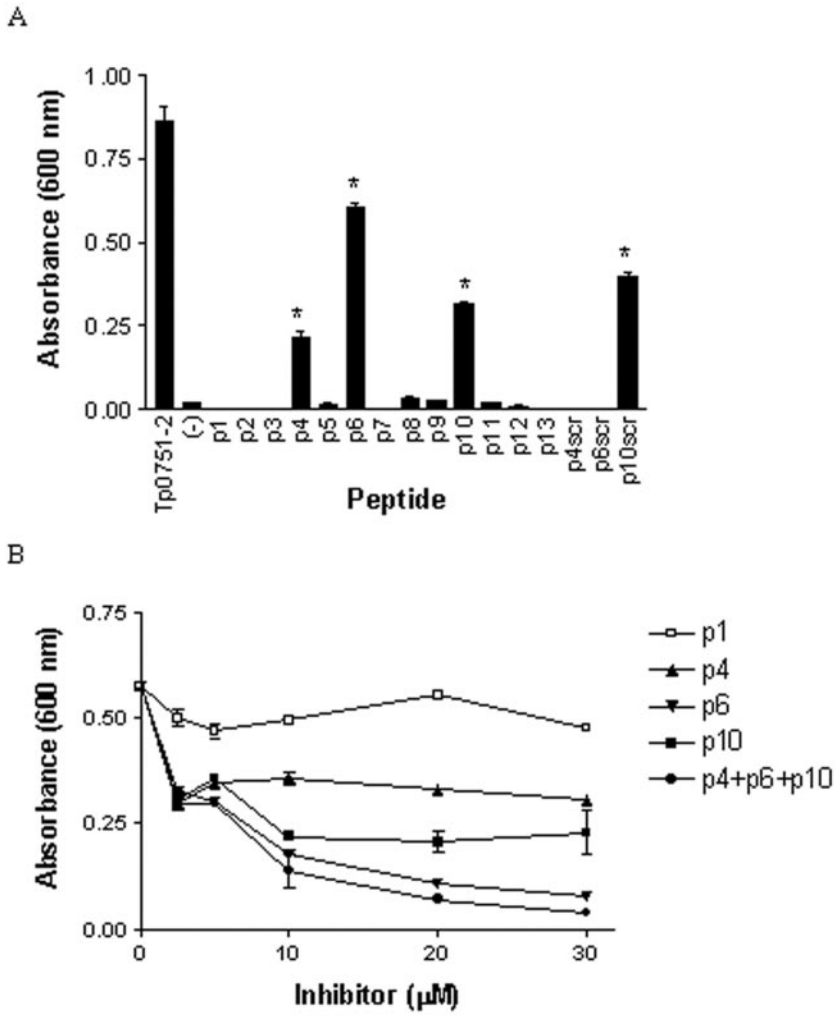

FIG. 3. Identification of the peptide sequences that mediate attachment of Tp0751 to laminin. (A) Attachment of Tp0751-2 and synthetic peptides 1 to 13 to laminin. Also shown is the attachment potential of scrambled versions of synthetic peptides 4, 6, and 10. For statistical analyses, attachment of each of the synthetic peptides was compared with attachment of the negative control recombinant protein Tp0557 by the Student two-tailed *t* test (*, $P \leq 0.0002$). (B) Peptide inhibition of Tp0751-2 attachment to laminin. The capacities of peptides 1, 4, 6, and 10 to inhibit attachment of Tp0751-2 to laminin were investigated.

were performed. In these studies, peptides 4, 6, and 10 were tested for their ability to inhibit the binding of Tp0751-2 to laminin. As shown in Fig. 3B, peptides 4, 6, and 10, when added individually, were able to inhibit attachment of Tp0751-2 to laminin in a concentration-dependent manner. When all three peptides were added together, complete inhibition was observed at the highest peptide concentration tested (30 μM). The peptide concentration at which 50% inhibition was observed (ID_{50}) varied for each of the peptides, with peptide 6 exhibiting the highest level of inhibition (ID_{50} = 6.7 μM) and peptides 4 and 10 displaying lower levels of inhibition (ID_{50} = >30 μM and ID_{50} = 7.1 μM, respectively). These results correlate well with the level of attachment to laminin observed for each of the peptides, with peptide 6 showing the highest degree of attachment and peptides 4 and 10 demonstrating lower levels of attachment (refer to Fig. 3A); this suggests that the amino acid residues present in peptide 6 contain the major laminin binding site of the Tp0751 molecule. No inhibition of laminin attachment was detected when Tp0751-2 was preincubated with a peptide that did not mediate laminin binding, namely peptide 1, thus demonstrating that the observed inhibition was peptide specific. These results complement the findings obtained from the direct peptide attachment studies and further indicate that peptides 4, 6, and 10 contain amino acid residues involved in the Tp0751-laminin interaction.

The observation that peptides 3, 5, 7, 9, and 11 did not exhibit any detectable binding to laminin suggests that the linear configuration of amino acids contained within these peptides does not support laminin attachment. Since each peptide includes 10 overlapping residues from the preceding and succeeding peptides (refer to the peptide design in Fig. 1), this implicates the four amino acids unique to each peptide as being part of the minimum sequence within Tp0751 that mediates laminin attachment. To verify this finding, mutagenized peptides were prepared that incorporated alanine or glycine

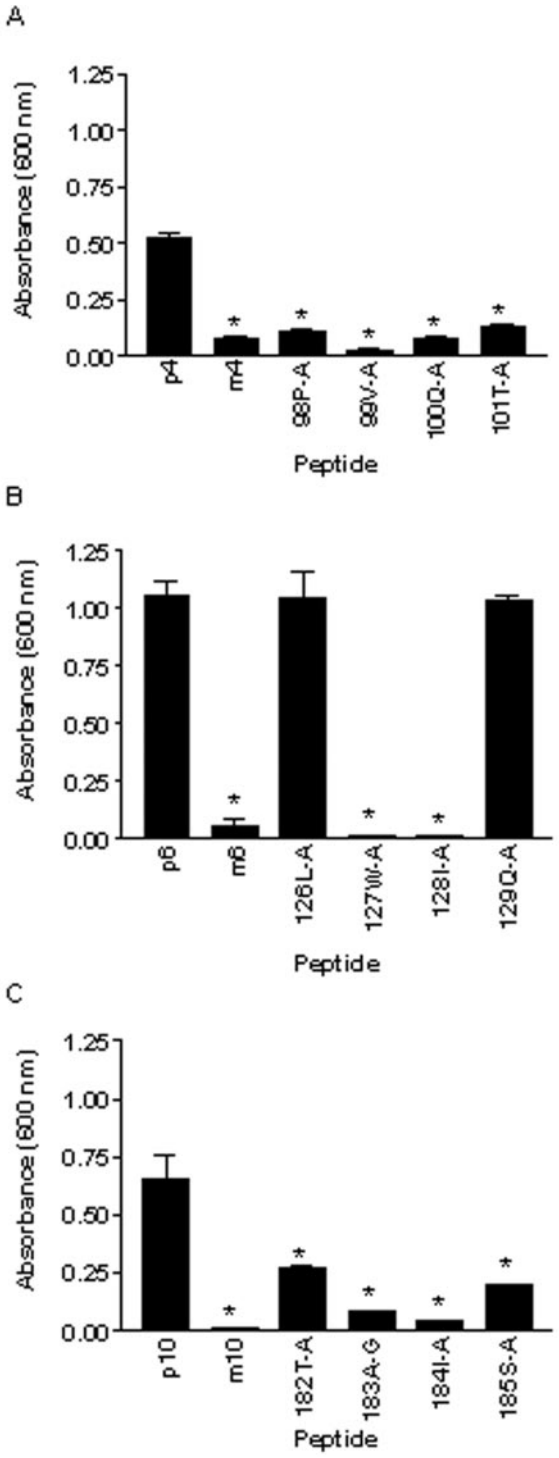

FIG. 4. Delineation of the amino acid residues mediating attachment of Tp0751 to laminin. Shown is the laminin attachment potential of native and mutant peptides for the sequences covered by peptide 4 (A), peptide 6 (B), and peptide 10 (C). Data are reported as the mean absorbances at 600 nm ± SEM for triplicate wells. Statistical analyses compared the level of attachment of each mutant peptide to that of the native peptide by the Student two-tailed *t* test (∗, $P < 0.0001$ [A and B] or $P \leq 0.0139$ [C]).

residues in place of the four amino acids that are unique to each of peptides 4, 6, and 10 (refer to Fig. 1). As shown in Fig. 4A to C, replacement of all four unique amino acids abolished the capacity of each peptide to attach to laminin. Subsequent investigations revealed that 10 amino acids are critical to the laminin attachment potential of the Tp0751-derived peptides, since mutagenesis of each of these residues resulted in elimination of the laminin attachment potential for the particular peptide. These residues included the four unique amino acids P^{98}, V^{99}, Q^{100}, T^{101} within peptide 4 (Fig. 4A), amino acids W^{127} and I^{128} within peptide 6 (Fig. 4B), and the four unique amino acids T^{182}, A^{183}, I^{184}, and S^{185} within peptide 10 (Fig. 4C). Mutagenesis of the other amino acids unique to peptide 6 had no effect on the laminin-binding capacity of the peptide (Fig. 4B), suggesting that these amino acid residues do not contribute to the ability of peptide 6 to bind to laminin.

Tp0751 cellular attachment potential. To determine whether Tp0751 could mediate attachment of laminin-producing mammalian cells, cellular adhesion assays were performed using wells coated with Tp0751-1 and the colon carcinoma cell line SW480. Increased cell surface expression of laminin is observed on cancer cells (75), thereby allowing for easy detection of the attachment level of such cells to laminin-binding proteins. As shown in Fig. 5, wells coated with Tp0751-1 demonstrated a significantly higher level ($P < 0.001$) of attachment of SW480 cells than did wells coated with the negative control protein BSA. The degree of binding observed was similar to that seen with wells coated with the positive control protein fibronectin, which mediates attachment of mammalian cells through the integrin receptor (32).

Inhibition of *T. pallidum* attachment to laminin. The ability of the Tp0751-derived peptides to inhibit the attachment of *T. pallidum* to laminin-coated surfaces was determined. Dark-field images documenting the results of these studies are shown in Fig. 6A, and panel B quantifies the number of treponemes attaching to laminin under each peptide condition. In these studies, laminin that had been preincubated with peptides 4, 6, and 10 inhibited attachment of *T. pallidum* to the laminin-coated slides by 91% compared to laminin that had undergone the same reaction conditions but with no peptide additions. Similar results were obtained when laminin was preincubated with each of the peptides individually (data not shown). Preincubation of laminin with peptides 1, 2, and 3 and the mutagenized versions of peptides 4, 6, and 10 (refer to Fig. 1) did not inhibit attachment of *T. pallidum* to laminin, nor did preincubation with each of these peptides individually (data not shown).

In further studies, the ability of anti-Tp0751 polyclonal serum to inhibit treponemal attachment to laminin-coated surfaces was determined. Representative dark-field images showing treponemal attachment upon preincubation with either anti-Tp0751 or control antiserum are shown in Fig. 7A, and the number of treponemes attaching to laminin under each condition is documented in Fig. 7B. In these experiments, *T. pallidum* that had been preincubated with anti-Tp0751 serum exhibited a significant reduction in attachment to laminin-coated slides, with 89.5% fewer treponemes attaching to the laminin-coated surface in the presence of this serum than with *T. pallidum* preincubated with the buffer diluent with no added

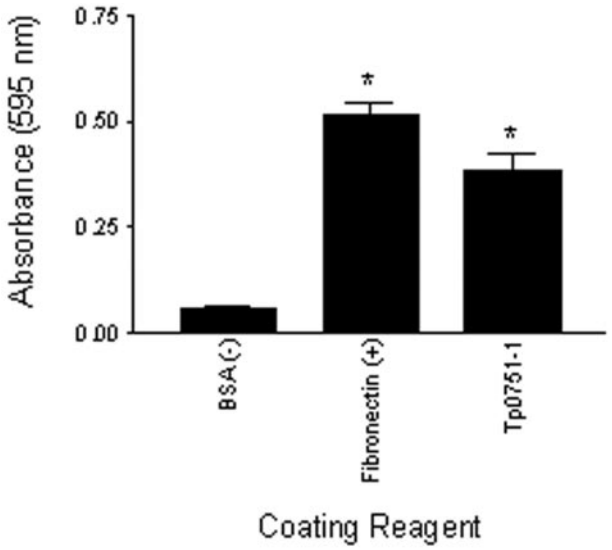

FIG. 5. Cellular attachment potential of recombinant Tp0751. Shown is attachment of the SW480 cell line to BSA (negative control), fibronectin (positive control), and Tp0751-1. Data are reported as the mean absorbance at 595 nm ± SEM for three wells. Statistical analyses compared the level of attachment to each cell line by the Student two-tailed *t* test (*, $P < 0.001$).

serum. Additional controls included *T. pallidum* preincubated with immune rabbit serum, normal rabbit serum, and the irrelevant anti-Tp0155 serum. Treponeme preincubation with the control immune rabbit serum resulted in 92.5% fewer treponemes attaching to the laminin-coated surfaces, while no (0%) and minimal (7%) inhibition of treponemal attachment to laminin was observed with preincubation with normal rabbit serum and anti-Tp0155 serum, respectively.

DISCUSSION

The natural history of syphilis includes four frequently overlapping stages: primary, secondary, latent, and tertiary syphilis. Bacterial dissemination from the site of inoculation occurs within the primary stage of infection, and late manifestations of the disease can involve any organ system, thus illustrating the highly invasive nature of *T. pallidum*. All stages of syphilis are characterized by vascular involvement, and in particular, *T. pallidum* localizes to perivascular areas in infected tissues (43, 58). Further, *T. pallidum* specifically attaches to vascular endothelium (37) and isolated basement membranes (22), and the organisms traverse endothelial cell monolayers by moving through the junctions between cells (20, 21, 31, 62, 68, 69). Additionally, two areas in which *T. pallidum* localizes include laminin-containing cutaneous nerves (65) and the dermal-epidermal junction within the perivascular area of the skin (5, 15). Two interrelated hypotheses that can be drawn from these observations are that *T. pallidum* specifically localizes within infected tissues to areas rich in laminin and that the proximity of *T. pallidum* to perivascular locales during infection would arise from and facilitate treponemal dissemination via the bloodstream. As such, laminin is likely to play a central role in *T. pallidum* pathogenesis, and therefore, interfering with the *T. pallidum*-laminin interaction may allow for alteration of the course of infection and prevention of treponemal dissemination and establishment of chronic infection.

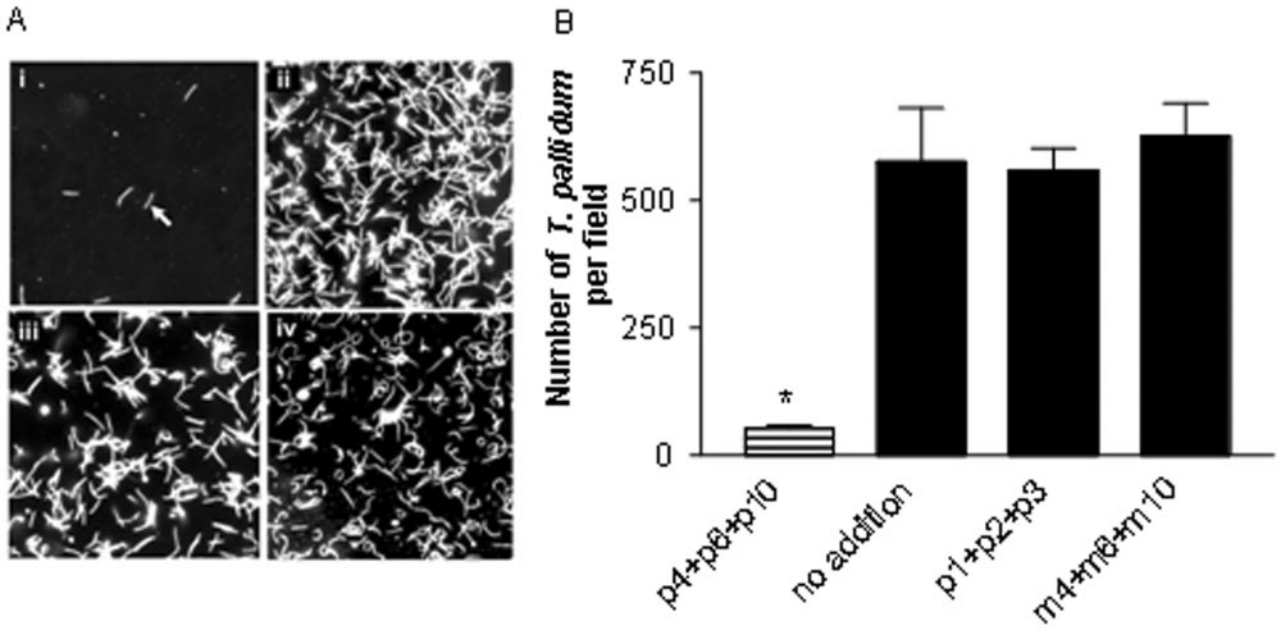

FIG. 6. Peptide inhibition of *T. pallidum* attachment to laminin. (A) treponemes were incubated with laminin-coated slides in the presence of peptides 4, 6, and 10 (i), diluent alone with no peptide addition (negative control, ii), control peptides 1, 2, and 3 (negative control, iii), and mutagenized peptides 4, 6, and 10 (m4+m6+m10; iv). A representative spirochete is identified by an arrowhead in panel i. Spirochetes were visualized by dark-field microscopy using a Nikon Eclipse E600 microscope. (B) Quantitation of the number of treponemes attached per field under each reaction condition. Statistical analyses compared the level of attachment of each condition to that of the "no addition" sample by the Student two-tailed *t* test (*, $P < 0.0001$).

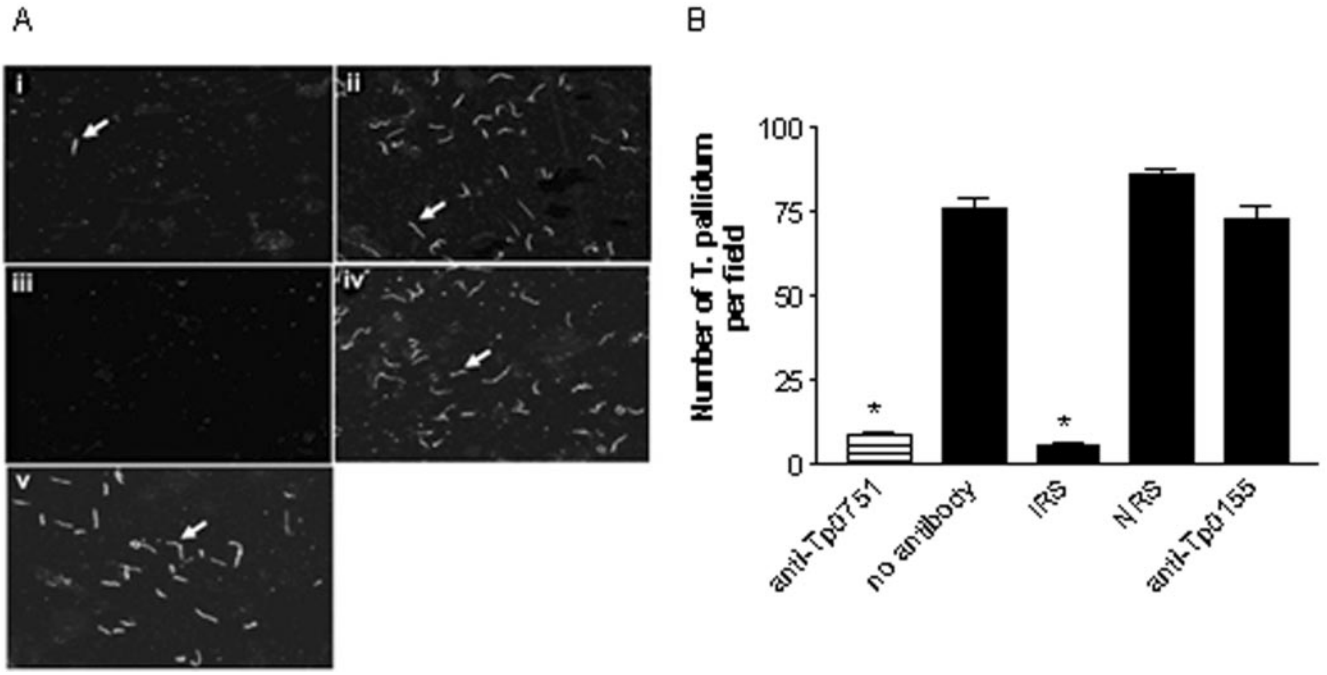

FIG. 7. Antibody inhibition of *T. pallidum* attachment to laminin. (A) treponemes were incubated with laminin-coated slides in the presence of anti-Tp0751-1 serum (i), diluent alone with no antibody addition (negative control, ii), immune rabbit serum (positive control, iii), normal rabbit serum (negative control, iv), and the irrelevant control serum anti-Tp0155 (negative control, v). Representative spirochetes are identified by arrowheads in panels i, ii, iv, and v. Spirochetes were visualized by dark-field microscopy using a Nikon Eclipse E600 microscope. (B) Quantitation of the number of treponemes attached per field under each reaction condition. Statistical analyses compared the level of attachment of each serum condition to that of the "no antibody" sample by the Student two-tailed *t* test (*, $P < 0.0001$).

Previous investigations identified Tp0751, a *T. pallidum* adhesin expressed during infection that exhibits specific attachment to laminin (7). In this report we expand upon these studies and further characterize the interaction of Tp0751 with laminin. Tp0751 was demonstrated to mediate attachment of cells expressing laminin, and further, antibodies specific for Tp0751 inhibited attachment of *T. pallidum* to laminin, thus confirming the identity of this *T. pallidum* protein as an adhesin. Through the use of synthetic peptides, we have identified 10 amino acids that are critical to the laminin attachment potential of Tp0751, including amino acids 98 to 101, 127 to 128, and 182 to 185, with the major laminin-binding epitope comprising residues 127 to 128. Peptides containing these sequences inhibited attachment of *T. pallidum* to laminin, while mutagenized versions of these peptides did not inhibit treponemal attachment. The interaction of residues 98 to 101 and 127 to 128 with laminin appears to be dependent upon conservation of the linear peptide sequence; supporting evidence includes the observations that these peptides inhibited attachment of Tp0751 to laminin, and scrambled versions of these peptides did not exhibit an affinity for laminin. Scrambled versions of the peptide encompassing residues 182 to 185 retained the ability to attach to laminin; this observation suggests that this region of Tp0751 requires merely the presence of those amino acids, and not the contiguous linear sequence, to interact efficiently with laminin.

The identification of the residues mediating Tp0751 attachment to laminin represents the first step in the process of devising an antiadhesive therapy to block bacterial attachment and dissemination. Multiple examples exist of bacterial adhesins that function as effective vaccine candidates, including the Hap adhesin from *Haemophilus influenzae* (42) and the collagen adhesin from *Staphylococcus aureus* (50). Peptide inhibitors have also been used to prevent adherence of selected bacteria to their attachment ligands, including the bacterial pathogens *Streptococcus mutans* (35) and *Porphyromonas gingivalis* (38) and the noscomial pathogen *Pseudomonas aeruginosa* (6). These studies have successfully prevented infection by targeting adhesins via multiple routes, including vaccination with recombinant protein antigens, therapeutic administration of selected peptides to competitively inhibit pathogen attachment, and passive administration of antibodies raised against peptide sequences mediating host attachment. As a result, these studies demonstrate the feasibility of developing antiadhesin vaccines and therapies.

Laminins are large heterotrimeric glycoproteins consisting of an α-, a β-, and a γ-type chain. To date, 5 α, 3 β, and 3 γ chains have been identified that give rise to at least 15 different laminin isoforms (16, 48, 70, 74). Although certain isoforms, such as laminin 10, show widespread distribution, other isoforms are functionally distinct and expressed in a tissue-specific and developmentally regulated manner, thus creating marked heterogeneity among basement membranes found throughout the host (3, 12, 77). For example, laminin 1 is expressed in tissues containing epithelial basement membranes (13), laminin 2 and 4 are components of skin, skeletal muscle, heart muscle, and nerve cells (29, 39), and laminin 8 is localized in adipocytes, muscle cells and tissues containing epithe-

lial and endothelial basement membranes (33, 55). The widespread distribution of laminin makes it an appropriate target for a disseminating pathogen such as *T. pallidum*, which has been shown to localize to a diverse range of these and other tissue sites. As shown in this report, Tp0751 efficiently attached to each of the laminin isoforms tested, including laminins 1, 2, 4, 8, and 10, thus strengthening the concept that laminin located in anatomically distinct tissue sites could function as a common target to allow attachment of *T. pallidum* to the host via Tp0751.

The intimate association of *T. pallidum* with the vascular endothelium is exemplified by the perivasculitis and endothelial cell abnormalities that are characteristically observed upon histopathologic analysis of infected tissues (45). Studies have shown that *T. pallidum* specifically activates endothelial cells (60, 61), and in turn, endothelial cell activation leads to increased vascular permeability (4, 73, 79). Laminin plays an important role in regulating endothelial cell morphogenesis (28), and we hypothesize that the specific interaction of the *T. pallidum* adhesin Tp0751 with laminin in the endothelial cell layer may facilitate treponemally induced endothelial cell activation and subsequent transendothelial treponemal passage to promote tissue invasion. Other situations which cause endothelial cell activation and in turn increased vascular permeability include tumor growth and chronic inflammation (46, 52) and infection with the pathogens *Escherichia coli* (67) and *Trypanosoma brucei* (26).

Significant insight can be gleamed from the study of mechanisms of dissemination of tumor cells and interaction of such cells with laminin. Tumor cells specifically attach to basement membranes, and laminin-binding proteins on tumor cell surfaces are crucial for metastasis (41). Tumor cells intravasate by penetrating the basement membrane, and although this step is incompletely understood, degradation of basement membrane components by proteolytic enzymes is proposed to be involved (80). Similarly, various invasive bacterial pathogens, including *Haemophilus influenzae*, *Salmonella enterica*, *E. coli*, and *Yersinia pestis*, adhere to laminin and initiate a proteolytic cascade that facilitates basement membrane degradation and bacterial invasion (36). Along these lines, the related spirochete *Treponema denticola* binds to extracellular matrix components (18, 19) and expresses a chymotrypsin-like protease that degrades basement membrane components and promotes treponemal invasion (14, 17, 30). Whether a similar situation contributes to the remarkable invasive capability of *T. pallidum* remains to be determined.

In summary, in this report we have determined that Tp0751 mediates attachment of mammalian cells expressing laminin and that this adhesin exhibits an attachment profile similar to those of multiple laminin isoforms that are widespread throughout the host. We have delineated the amino acid residues involved in attachment of the *T. pallidum* adhesin Tp0751 to laminin and have identified the minimum laminin-binding epitope for attachment. Future investigations will target these identified laminin-binding regions of Tp0751 to determine if in vivo inhibition of treponemal attachment to laminin alters the course of *T. pallidum* infection, dissemination, and disease progression.

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