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Molecular cloning of *Treponema pallidum* outer envelope fibronectin binding proteins, Pl and P2

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SUMMARY Phages directing the synthesis of *Treponema pallidum* fibronectin binding adhesin proteins, Pl and P2,[1] were isolated from an EMBL-3 bacteriophage lambda library of *T pallidum* deoxyribonucleic acid (DNA). The recombinant phages were identified using antisera generated to treponemal proteins purified in fibronectin-Sepharose. Recombinant Pl and P2 proteins possessed the same relative molecular weights as the native surface polypeptides of spirochaetes. The structural genes for these proteins were subcloned into the plasmid vector pUC19, and transformed *Escherichia coli* expressed and translocated recombinant Pl and P2 to their outer membranes. Finally, the recombinant adhesin proteins, Pl and P2, were purified from detergent solubilised *E coli* outer membrane preparations using fibronectin-Sepharose affinity chromatography, which confirmed that the fibronectin binding properties of the cloned proteins were retained.

The spirochaete that causes syphilis, *Treponema pallidum*, attaches by a specialised tip structure[2] to both eucaryotic cells and extracellular matrix by the selective recognition of host fibronectin.[1 3 4] Three treponemal proteins designated Pl (molecular weight 89 300 daltons), P2 (37 000 daltons), and P3 (32 000 daltons), which are located on the spirochaete outer envelope, have been implicated as putative adhesins,[5 6] and have been purified subsequently by fibronectin-Sepharose affinity chromatography.[1]

The inability to cultivate large quantities of *T pallidum* organisms has impeded our attempts to identify the functional domains of the treponemal adhesins that recognise host fibronectin.[7] Recombinant deoxyribonucleic acid (DNA) techniques provide a means of obtaining sufficient quantities of specific treponemal proteins to purify and subsequently characterise them.[8–11]

Because the fibronectin binding proteins of *T pallidum* represent the only treponemal surface proteins with assigned biological properties,[1 3] the availability of recombinant adhesins offers opportunities for testing rational vaccines and diagnostic probes for syphilis and other treponematoses. In this study we describe the molecular cloning of the structural genes for the fibronectin binding adhesin proteins of treponemes. We discuss the availability of the recombinant adhesins for the future understanding of the complex interaction between host and parasite and of the pathogenesis of syphilis.

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Materials and methods

BACTERIA

Treponema pallidum organisms were maintained by serial passage in the testes of New Zealand white rabbits, and treponemes were harvested from testicular tissue as described previously.[2 6 9] Spirochaetes separated from host material were used for attachment assays at densities of 1×10^9 organisms/ml.[1 3] About 4×10^9 motile spirochaetes in 10 ml of treponemal medium were radiolabelled with 2·0 mCi of radiosulphated (^{35}S) methionine (specific activity, 1500 Ci/mmole; Amersham Corporation, Arlington Heights, Illinois, USA) for two hours at 34°C under aerobic conditions.[1 6] Radiolabelled organisms were then pelleted at 15 000 × *g* and resuspended in phosphate buffered saline (PBS) for use in attachment assays.

E coli strains LE392[12] (F; *hsdR* 514, *supE* 44, *supF* 58, *lacY*, *galK* 2, *galT* 22, *metB*, *trpR* 55, *lambda*$^-$)

and TB1[12] (*Δ lac-pro, strA, ara, thi, φ*80dlacZ, *Δ* M15 *hsdR*) were grown in Luria broth.[12]

ANTISERA

Serum from syphilitic rabbits was obtained from intratesticularly inoculated rabbits housed for at least 40 days before infection,[13] and was extensively adsorbed against *E coli* before its use in immunoblots of recombinant *E coli* proteins.[9 11] Antisera to treponemal fibronectin binding proteins designated P1, P2, and P3 were prepared by immunising rabbits[7] with fibronectin-Sepharose purified treponemal proteins.[1] These antisera were adsorbed with an extract of *E coli* obtained in a French pressure cell apparatus[9] and with fibronectin coupled to Sepharose[1] so that no reactivity existed for *E coli* proteins or fibronectin. IgG fractions of these antisera were prepared by protein A-Sepharose affinity chromatography. Serum from patients with syphilis was provided by Sandra Larsen of the Centers for Disease Control, Atlanta, Georgia.[14] Serum samples from healthy rabbits and from people without a history of sexually transmitted diseases were used as controls.

ATTACHMENT OF *T PALLIDUM* TO FIBRONECTIN COATED COVERSLIPS AND HEp-2 CELLS

Glass coverslips coated with fibronectin were prepared as described previously.[1-3] Human epithelial cells (HEp-2; ATCC, Rockville, Maryland, USA) were passaged in Dulbecco's minimum essential (DME) medium supplemented with 10% fetal calf serum, and 5×10^4 HEp-2 cells were seeded in Leighton tubes containing individual 9×35 mm coverslips for 24 hours before attachment assays.

Freshly extracted *T pallidum* organisms or intrinsically labelled spirochaetes were treated with IgG antibody (500 mg/l) for one hour before being incubated for two hours at 34°C with fibronectin coated coverslips or HEp-2 cells.[1-3] The coverslips were then removed and rinsed nine times by immersion in a beaker containing PBS before each was placed in a vial with scintillation fluid to determine adherent radioactivity.[1 3 6]

AFFINITY PURIFICATION OF PROTEINS P1, P2, AND P3 WITH FIBRONECTIN-SEPHAROSE

Adhesin proteins from *T pallidum* organisms were isolated by fibronectin-Sepharose affinity chromatography.[1] Recombinant proteins P1 and P2 were purified similarly using outer membranes of transformed *E coli*. Briefly, 1 ml containing about 100 mg of *E coli* outer membranes prepared as outlined below was solubilised adding 100 μl of 10% Zwittergent 3–12 (Z(3–12); Calbiochem-Boehring). This extract was then homogenised and diluted in PBS to give a final Z(3–12) detergent concentration of 0·05% for chromatography.[1] *E coli* proteins adherent to fibronectin were released by boiling the extract in electrophoresis dissolving buffer, and were used in immunoblotting.

IMMUNOLOGICAL SCREENING OF *T PALLIDUM* DNA CLONE BANK PREPARED BY Bam HI AND Sal I DIGESTION

A clone bank prepared in bacteriophage λ EMBL-3 using *T pallidum* DNA partially digested by Bam HI was described in an earlier study.[9 11] A clone bank of *T pallidum* DNA treated with Sal I was also constructed similarly. Recombinant phages were plated on *E coli* LE392 to produce about 500 plaques per plate. The plates were overlaid with nitrocellulose discs, which were incubated at 4°C overnight, removed, and screened with antisera prepared against *T pallidum* proteins adherent to fibronectin.[1 3] Clones producing signals were screened again in duplicate for positive reactions, and were immunoblotted.

CONSTRUCTION OF PLASMIDS CONTAINING P1 AND P2 STRUCTURAL GENES

Recombinant phages producing a recombinant treponemal protein reactive with specific antisera to the treponemal fibronectin binding proteins were amplified and purified from two L cultures of *E coli* LE392. The phage DNA was isolated[12] and enzymatically digested with Bam HI (P1 encoding phage) and Sal I (P2 encoding phage). Insert fragments of 6·6 kb for P1 and 5·2 kb for P2 were ligated individually into the respective Bam HI or Sal I sites of the plasmid vector, pUC19. Ligated DNA was then used to transform *E coli* TB1. Colonies displaying a white phenotype were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to express P1 and P2 proteins.

FRACTIONATION OF *E COLI* CELLS

The subcellular location of recombinant P1 and P2 was established by differential centrifugation of *E coli* treated in a French pressure cell apparatus.[15] One litre of *E coli* growing at the mid-logarithmic phase was harvested by centrifugation and washed once with 10 mmol/l N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES) buffer, pH 4. The bacteria were resuspended in 30 ml HEPES buffer and treated at 20 000 psi on a French pressure cell apparatus. The membrane fractions were then separated by centrifugation as described previously.[9 11 15]

SDS-PAGE AND IMMUNOBLOTTING

SDS-PAGE and immunoblotting of *T pallidum* or *E coli* proteins have been described previously.[1 5 6] Total trichloroacetic acid protein preparations of *T*

pallidum were prepared for electrophoresis and immunoblotting as detailed elsewhere.[6]

Results

SPECIFICITY OF ANTISERA GENERATED AGAINST *T PALLIDUM* FIBRONECTIN BINDING PROTEINS

It was important first to establish the specificity of antibodies generated in rabbits to *T pallidum* adhesins P1, P2, and P3 purified by fibronectin-Sepharose chromatography.[1] Using an extract of detergent solubilised *T pallidum* that contained numerous treponemal proteins (fig 1, lane A), the selective immunodetection of the previously identified adhesins was shown (lane C).[1] In contrast, syphilitic rabbit serum recognised many additional *T pallidum* polypeptides in a duplicate blot of the same extract (lane B). Antisera to P1, P2, and P3 failed to recognise proteins of the non-pathogenic spirochaete, *Treponema phagedenis* biotype Reiter (data not shown).

Incubating of *T pallidum* with the IgG fraction of syphilitic rabbit serum or with antisera to P1, P2, and P3 proteins before adding treponemes to coverslips coated with fibronectin or to HEp-2 cell monolayers[1 3] decreased treponemal binding by more than half. Treating organisms with normal rabbit IgG did not diminish binding to fibronectin or to HEp-2 cells, as we have reported previously.[1-3 6]

PHAGE CLONES EXPRESSING *T PALLIDUM* PROTEINS P1 AND P2

An EMBL-3 phage library obtained from *T pallidum* DNA digested with Bam HI and Sal I was screened with antisera to treponemal proteins that bind fibronectin. Phages producing positive signals were cloned and their lysates analysed by immunoblot using pooled serum from people with syphilis. Two distinct patterns of immunoreactivity were detected (fig 2). We observed recombinant proteins that comigrate with P1 (lane B1) and with P2 (lane C1). Lane A shows immunoblot profiles of proteins P1, P2, and P3 using total *T pallidum* proteins and the antisera to treponemal proteins that bind fibronectin. Plaque purified antibodies[9] recognised the same proteins (lanes B2 and C2, respectively), which suggested that the structural genes for these two *T pallidum* proteins were entirely cloned.

PLASMID EXPRESSION OF P1 AND P2 IN *E COLI*

To facilitate further analysis of recombinant P1 and P2, we subcloned their respective structural genes into the multicopy expression plasmid, pUC19. The phage derived *T pallidum* insert DNA was ligated into pUC19, and an attempt was made to transform *E coli*. Colonies displaying a white phenotype were screened by immunoblot using antisera to P1, P2, and P3. We isolated clones that expressed the treponemal adhesin proteins, P1 and P2.

Finally, we identified the cellular location of P1 and P2 in *E coli* transformed by pUC19. Figure 3 shows recombinant proteins P1 and P2 in both the cytoplasmic and outer membrane fractions of recombinant *E coli*.

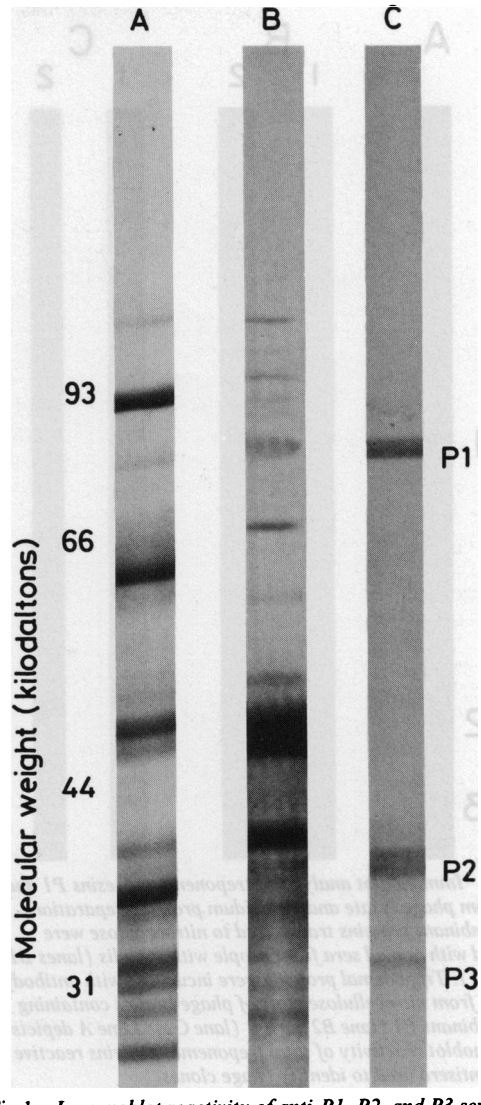

Fig 1 *Immunoblot reactivity of anti-P1, P2, and P3 sera to total Treponema pallidum proteins. Proteins were stained with Coomassie brilliant blue (lane A) or electrophoretically transferred to nitrocellulose and incubated with syphilitic rabbit sera (lane B) or sera from rabbits immunised with T pallidum fibronectin binding proteins (lane C).*

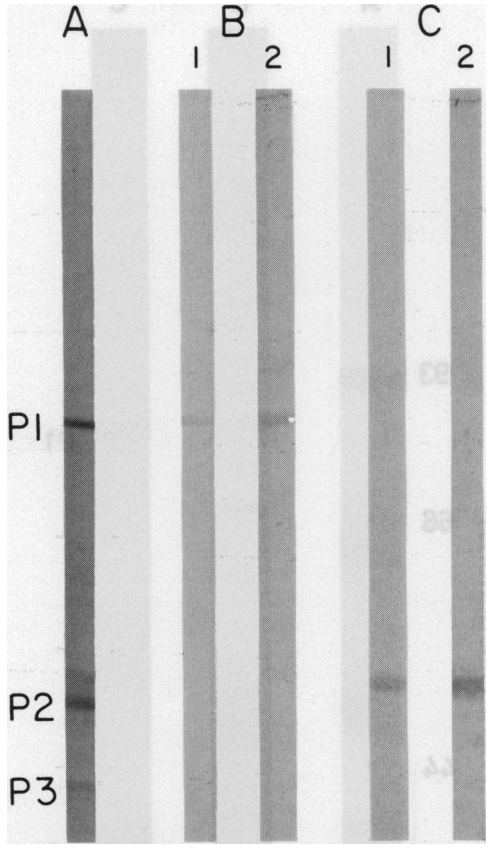

Fig 2 *Immunoblot analysis of treponemal adhesins P1 and P2 from phage lysate and T pallidum protein preparations. Recombinant proteins transferred to nitrocellulose were probed with pooled sera from people with syphilis (lanes B1 and C1). Treponemal proteins were incubated with antibody eluted from nitrocellulose blots of phage lysates containing recombinant P1 (lane B2) or P2 (lane C2). Lane A depicts immunoblot reactivity of total treponemal proteins reactive with antisera used to identify phage clones.*

AFFINITY PURIFICATION OF RECOMBINANT P1 AND P2 USING FIBRONECTIN-SEPHAROSE

To demonstrate the fibronectin binding ability of recombinant P1 and P2, a Z(3–12) detergent extract of outer membranes of recombinant *E coli* expressing either P1 or P2 was chromatographed on fibronectin-Sepharose. Analysis by immunoblot of adherent material using antisera to proteins P1, P2, and P3 showed the presence of recombinant P1 or P2 (fig 4, lanes A1 and B1), which comigrated with P1 and P2 purified from *T pallidum* detergent extracts. Non-adherent material evaluated similarly (fig 4, lanes A2 and B2) failed to react in the immunoblot

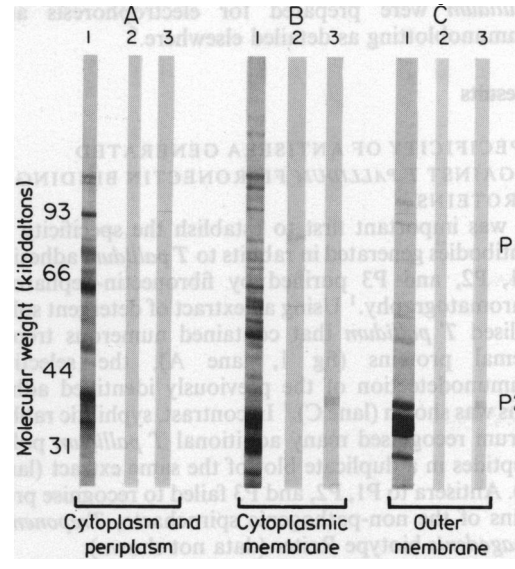

Fig 3 *Immunoblot localisation of P1 and P2 in transformed Escherichia coli. Recombinant E coli cells were treated in French pressure cell apparatus and separated into cytoplasm and periplasm, cytoplasmic membrane, and outer membrane fractions. Protein fractions were electrophoresed and stained with Coomassie brilliant blue (lanes A1, B1, and C1) or blotted on to nitrocellulose and incubated with syphilitic rabbit serum (lanes A2, A3, B2, B3, C2 and C3).*

assay. As an additional control in the experiment, an outer membrane preparation of *E coli* containing a plasmid expressing a 70 000 dalton treponemal protein not recognised by antisera to the fibronectin binding proteins (P1, P2, and P3) was detected only in the non-adherent fraction when probed with syphilitic rabbit serum (fig 4, lanes C1 and C2). Under similar experimental conditions fibronectin-Sepharose chromatography of a total detergent extract of *T pallidum* showed the selective purification and immunoreactivity of P1, P2, and P3.

Discussion

Molecular examination of the interactions of *T pallidum* with eukaryotic cell surfaces identified three outer envelope treponemal proteins as putative ligands.[1 5 6] In separate studies examining treponemal acquisition of host macromolecules, the putative adhesins showed high affinity for fibronectin.[1 3] Additionally, antibodies to fibronectin but not to other components of the extracellular matrix greatly diminished treponemal parasitism of host cells.[3] Other observations reinforced the idea of receptor to ligand specificity for interactions between *T pallidum* and host cells.[1–3 6]

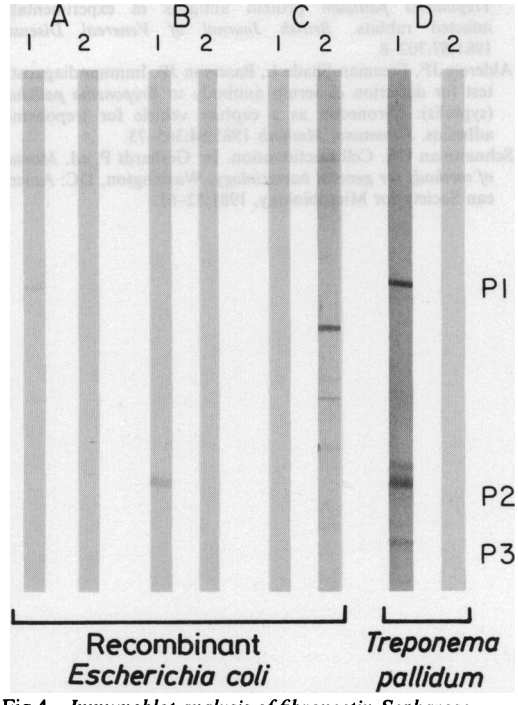

Fig 4 *Immunoblot analysis of fibronectin-Sepharose chromatographed protein preparations. Recombinant E coli outer membrane proteins (lanes A to C) and T pallidum organisms (lane D) were detergent solubilised and chromatographed on fibronectin-Sepharose affinity columns. Profiles of immunoblots with syphilitic rabbit serum of adherent fractions from E coli producing P1 (lane A1), P2 (lane B1), and a 70 kilodalton treponemal protein (lane C1) and T pallidum (lane D1). Lanes A2, B2, C2, and D2 show immunoblot profiles of non-adherent fractions from the same protein preparations.*

Using fibronectin-Sepharose affinity purified treponemal preparations we were able to generate antibodies with specificity for *T pallidum* proteins P1, P2, and P3 (fig 1). These antibodies inhibited attachment of treponemes to fibronectin and to HEp-2 cells and were used to screen the *T pallidum* genomic library[12] for expressing these biologically important peptides. Distinct phage clones directing the synthesis of 89 300 dalton (P1) and 37 000 dalton (P2) proteins were isolated. The treponemal origin of the structural genes of these proteins was shown by the ability of antibodies purified from blots of phage lysates to react with native *T pallidum* proteins of identical molecular weight (fig 2).

The respective phage DNAs encoding P1 and P2 were subcloned into the expression plasmid, pUC19. The resulting recombinant plasmids directed the expression of these proteins in transformed *E coli*. Furthermore, the plasmid encoded proteins were transferred to *E coli* outer membranes (fig 3). Fibronectin chromatography of detergent solubilised *E coli* outer membranes showed the affinity of recombinant P1 and P2 for fibronectin (fig 4), which indicated that the recombinant proteins, P1 and P2, retained functional integrity and can be used to identify their putative common fibronectin binding domain.[7]

Furthermore, immunoblot analysis of the recombinant adhesins with sera from people with syphilis (fig 2, lanes B1 and C1) reinforced reports that detailed the immunogenicity of P1 and P2.[2 5 6 13] This evidence provides the basis for molecular dissection of immunodominant epitopes in the native adhesins using recombinant peptides. Whether immune reactivity to these important functional immunogens is humoral or cellular may now be decided. Such information may also assist in clarifying the role of immune processes in treponemal infections.

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