

## Genetic and Immunological Analysis of *Mycobacterium tuberculosis* Fibronectin-Binding Proteins

CHRISTIANE ABOU-ZEID,<sup>1\*</sup> THOMAS GARBE,<sup>2</sup> RAJU LATHIGRA,<sup>2</sup> HARALD G. WIKER,<sup>3</sup>  
MORTEN HARBOE,<sup>3</sup> GRAHAM A. W. ROOK,<sup>1</sup> AND DOUGLAS B. YOUNG<sup>2</sup>

Department of Medical Microbiology, University College and Middlesex School of Medicine, Ridinghouse Street, London W1P 7PN,<sup>1</sup> and MRC Tuberculosis and Related Infections Unit, RPMS, Hammersmith Hospital, London W12 0HS,<sup>2</sup> United Kingdom, and Institute of Immunology and Rheumatology, University of Oslo, 0172 Oslo 1, Norway<sup>3</sup>

Received 13 February 1991/Accepted 21 May 1991

**Recombinant phage clones, TB1 and TB2, were selected from a *Mycobacterium tuberculosis* *lgt11* DNA expression library by screening with a polyclonal antiserum raised against the antigen 85 complex of *Mycobacterium bovis* BCG. Analysis of recombinant DNA inserts and expressed fusion proteins showed that two new genes had been isolated. The product of clone TB2 was identified as a member of the 30/31-kDa antigen 85 complex. Restriction enzyme analysis showed that this gene differs from previously cloned members of this antigen complex, with detailed serological analysis indicating that it may encode the 85C component. Antisera raised against the expressed product of clone TB1 recognized a 55-kDa protein in *M. tuberculosis* extracts. The 55-kDa protein also has fibronectin-binding activity and, like the 30/31-kDa family, is a prominent target of the antibody response in patients with mycobacterial disease. Although the clones were selected by using the same antiserum, detailed analysis by serology and by DNA hybridization showed that they represent two quite distinct types of fibronectin-binding activities expressed by *M. tuberculosis*. Further analysis of the fibronectin-binding antigens of *M. tuberculosis* may provide important insights into their role in mediating the interaction with the host immune system.**

*Mycobacterium tuberculosis* remains a major and complex pathogen to humans because of its ability to survive the host response within phagocytic cells and to evoke tissue-damaging immunopathology. Elucidation of the mechanisms involved at a molecular level could lead to novel vaccines and drugs. An important research effort has been made over the last 5 years to identify components of mycobacteria which are involved in interaction with the host immune system. By using monoclonal antibodies and oligonucleotide probes based on peptide sequences derived from isolated proteins, many genes encoding protein antigens have been cloned from *M. tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium bovis* BCG (for a review, see reference 33). Several of the major antigens have been found to belong to highly conserved stress protein families, and their role has been extensively discussed.

We have been interested in antigens secreted by live *M. tuberculosis*. Proteins which are secreted by mycobacteria during growth may become available for immune recognition at an early stage of infection and may therefore be relevant for protective immunity. Thus, we have identified radiolabeled proteins in short-term culture supernatants (2), which indicates that these proteins are being released by actively metabolizing organisms rather than leaking from dead ones. One group of proteins, constituting 20 to 30% of all proteins in supernatants of short-term cultures, are seen as a prominent doublet of 30 and 31 kDa on Western blots (1a) and correspond to the antigen complex termed BCG 85 in the crossed immunoelectrophoresis (CIE) reference system described by Closs et al. (6). This complex comprises at least three closely related components, namely, 85A, 85B, and 85C, which have extensive overlap in antibody recognition and N-terminal amino acid sequence but distinct structural

differences (20, 32). Nagai et al. (20) have recently found that a 27-kDa protein isolated from the culture supernatant of *M. tuberculosis* is related to the antigen 85 complex. Genes encoding the 85A component have been cloned from *M. tuberculosis* (5) and *M. bovis* BCG (9), while genes for 85B (also referred to as the  $\alpha$  antigen) have been cloned from *M. bovis* BCG (19) and from *Mycobacterium kansasii* (18). Sequence analysis shows the presence of a signal peptide in each of the genes which is absent from the mature protein, and the 85B component of *M. kansasii* has been exploited in the development of a secretion system for expression of foreign genes in mycobacteria (18). Sequence data also indicated that there is 77.5% homology between the 85A and 85B components but almost complete identity among the 85A components of *M. tuberculosis* and *M. bovis* BCG.

Current interest in this antigen complex was markedly enhanced by the demonstration of fibronectin (FN) binding to the 30/31-kDa proteins (1a). In a cell-associated form, this family of proteins can mediate attachment of whole bacteria to FN-coated surfaces (1a, 22). Moreover, we also identified a group of FN-binding proteins with a larger molecular mass of around 55 kDa (1a), although its relationship to the BCG 85 complex was unclear. FN is a glycoprotein found in plasma, in the extracellular matrix, and on many cell surfaces and bears binding sites for collagen, gelatin, fibrin, heparin, and cells (for a review, see Ruoslahti [24]). The ability of several pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, and some *Treponema* species, to bind to FN has been suggested to play a role in their virulence by promoting adherence to mucosal surfaces (4, 10, 27, 29), and FN-binding bacterial constituents may alter the functions of macrophages and the interactions of many cell types with the extracellular matrix.

To carry out detailed molecular analysis of the regulation and role of FN-binding proteins in mycobacterial pathogenesis, we have used a polyclonal antiserum raised against the

\* Corresponding author.

TABLE 1. Bacterial strains, phages, and plasmids

Bacteria, phage, or plasmid	Characteristics	Source <sup>a</sup>
<b>Bacteria</b>		
<i>M. tuberculosis</i> H37Rv	Clinical isolate	Department of Microbiology, UCMSM, London Glaxo Pharmaceuticals
<i>M. bovis</i> BCG	Vaccine strain	
<i>E. coli</i> Y1090	Propagation of $\lambda$ gt11	14
<i>E. coli</i> Y1089	Expression of $\lambda$ gt11 recombinants as lysogens	
<i>E. coli</i> BNN97	$\lambda$ gt11 lysogen	
<i>E. coli</i> JM105	Transformation recipient for plasmids	
<b>Phage</b>		
<i>M. tuberculosis</i> $\lambda$ gt11 library	Genomic DNA library	34
$\lambda$ TB1	$\lambda$ gt11 clone expressing <i>M. tuberculosis</i> 55-kDa FN-binding protein	This study
$\lambda$ TB2	$\lambda$ gt11 clone expressing <i>M. tuberculosis</i> 30-kDa FN-binding protein (85C)	This study
<b>Plasmid</b>		
pGEX-1	Expression vector	26
pGEXTB1	pGEX construct containing <i>Eco</i> RI insert from $\lambda$ TB1	This study
pGEXTB2	pGEX construct containing <i>Eco</i> RI insert from $\lambda$ TB2	This study

<sup>a</sup> UCMSM, University College and Middlesex School of Medicine.

BCG 85 complex to select relevant genes from a  $\lambda$ gt11 expression library. In this article, we report the cloning, expression, and characterization of a gene encoding a new member of the antigen 85 complex and also of a gene encoding a 55-kDa FN-binding antigen of *M. tuberculosis*.

## MATERIALS AND METHODS

**Bacterial strains, phages, and plasmids.** The bacteria, phages, and plasmids used in this study, as well as their properties, are outlined in Table 1.

**Mycobacterial antigens.** *M. tuberculosis* H37Rv and *M. bovis* BCG were grown for 21 days as surface pellicles on Sauton medium, and bacteria were then removed by filtration and sonicated as described previously (1a). Both culture supernatants and sonicates were passed through membrane filters (Millipore Corp.; pore size, 0.2  $\mu$ m).

**Production of antisera and monoclonal antibodies.** Polyclonal rabbit antiserum against the antigen 85 complex of *M. bovis* BCG and rabbit antisera to the purified A, B, and C components were produced as described previously (31). For the production of antibodies to glutathione *S*-transferase (GST)/TB1 and GST/TB2 fusion proteins, 50  $\mu$ g of purified antigens emulsified in 0.2 ml of incomplete Freund adjuvant was injected intramuscularly into BALB/c mice. Mice were boosted twice at 20-day intervals and bled 2 weeks afterwards. Sera were absorbed with a crude extract of an *Escherichia coli* strain which overexpressed an unrelated GST fusion protein in order to remove antibodies to GST and possible contaminating *E. coli* proteins. Monoclonal antibody HYT27 (3) was a generous gift from Ase Andersen and Jorgen Bennedsen, Statens Serum Institut, Copenhagen, Denmark.

**FN.** Affinity-purified human plasma FN was purchased from the New York Blood Center, New York, N.Y.

**Screening of  $\lambda$ gt11 *M. tuberculosis* recombinant DNA library with anti-BCG 85 antiserum.** A library prepared from randomly sheared genomic DNA of *M. tuberculosis* (Erdman strain) in the  $\lambda$ gt11 expression vector (34) was provided by R. A. Young, Whitehead Institute for Biomedical Research, Cambridge, Mass. The library was screened by using the rabbit anti-BCG 85 antiserum preabsorbed with *E. coli*

lysate as described previously (14, 34), with some minor modifications. Filters were blocked with 1% bovine serum albumin in phosphate-buffered saline (pH 7.2) with 0.05% Tween 20, incubated with the antibody at a 1:100 dilution overnight at 4°C, and probed with peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO P217) at a 1:1,000 dilution for 2 h at room temperature. Finally, the filters were developed with 4-chloro-1-naphthol. Recombinant phages producing positive signals were cloned by plaque purification with two cycles of isolation from single plaques.

**Preparation of crude *E. coli* lysates.** Lysogenic strains were obtained by infecting *E. coli* Y1089 with  $\lambda$ gt11 recombinant phages as described previously (14). Lysogens were grown in Luria-Bertani (LB) medium at 30°C to an  $A_{600}$  of 0.5. Cultures were then induced by incubation at 45°C for 20 min followed by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.) to 10 mM and further incubation at 37°C for 2 h. Harvested bacteria were resuspended in 1/20 of the original culture volume in phosphate-buffered saline, lysed, and stored at -20°C until used. Control lysate was prepared from strain BNN97, a lysogen prepared from  $\lambda$ gt11 phage without insert DNA.

**Recombinant DNA analysis.** Phage DNA was liberated from plate lysates amplified in *E. coli* Y1090 and purified by cesium chloride density gradient centrifugation (8). Plasmid DNA was prepared by using standard procedures (17). Mycobacterial DNA inserts were characterized from phage and plasmid preparations by digestion with restriction enzymes and analysis of fragments by electrophoresis on 0.7 or 1% agarose gels (17). Sizes of insert fragments were determined by running *Hind*III-digested  $\lambda$  DNA as size standards. Insert DNA was recovered after *Eco*RI digestion and gel electrophoresis by excision of bands from the gel and purification by using a GeneClean kit (Bio 101 Inc., La Jolla, Calif.).

**Southern blot analysis.** DNA probes for hybridization were labeled with [ $\alpha$ -<sup>32</sup>P]dATP (Amersham) by use of a random primed labeling kit (Boehringer Mannheim GmbH), and nonincorporated label was removed by passing the probes through a Nick column (Pharmacia). Southern blot and hybridization analyses were performed by using nylon mem-

branes (Hybond-N; Amersham) under conditions of high stringency as described previously (17).

**Overexpression of TB1 and TB2 gene products.** Vector pGEX-1 (26) was used to express the products of TB1 and TB2 at a high level in the form of GST fusion proteins. *EcoRI* DNA inserts from  $\lambda$ TB1 and  $\lambda$ TB2 were subcloned into the *EcoRI* site of vector pGEX-1, resulting in the construction of plasmids pGEXTB1 and pGEXTB2. After plasmid transformation of *E. coli* JM105 (11) and plating of the cells on LB medium containing 50  $\mu$ g of ampicillin per ml, single colonies were picked and inoculated in 1 ml of LB medium-ampicillin to select positive clones. Cultures were incubated overnight at 37°C, diluted 1:100 in 2 ml of fresh medium, and grown for 3 h at 37°C before isopropyl- $\beta$ -D-thiogalactopyranoside was added to a concentration of 0.1 mM. After an additional 2 to 3 h of growth, cells were pelleted and resuspended in 1/100 culture volume in distilled water.

GST fusion proteins were purified from 500 ml of induced cultures of *E. coli* JM105 transformed with pGEXTB1 and pGEXTB2 by use of the protocol described by Smith and Johnson (26). Briefly, Triton X-100 (BDH Chemicals) was added to 1% to lysed cells, and supernatants were passed through a glutathione-agarose column (Sigma). After the column was washed with phosphate-buffered saline (pH 7.3), fusion proteins were eluted by competition with free glutathione. GST was also prepared for use as a control and was purified from cells transformed with pGEX-1 without insert DNA.

**SDS-PAGE and Western blotting.** Protein antigen samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system (16) on slab gels of 12 or 6% acrylamide. A mixture of standard protein markers (MW-SDS-200; Sigma) was used for the determination of molecular mass.

For immunoblotting analysis, proteins were transferred from polyacrylamide gels onto nitrocellulose membranes as described previously (28). Localization of proteins was achieved by staining molecular weight markers and reference lanes with India ink (1  $\mu$ l/ml) in phosphate-buffered saline containing 0.3% Tween 20 (12). Processing of nitrocellulose blots with polyclonal or monoclonal antibodies was carried out as described above for immunoscreening of the  $\lambda$ gt11 library. FN-binding proteins were identified by incubating blots with FN (100  $\mu$ g/ml) for 2 h at 37°C and probed with peroxidase-conjugated rabbit anti-FN immunoglobulin (DAKO P246) at a dilution of 1:1,000 for 2 h at room temperature. To test the immune responses of patients and healthy controls to recombinant proteins, blots of *E. coli* lysates were screened with serum samples diluted between 1:100 and 1:500. Bound antibody was revealed by incubation with peroxidase-conjugated rabbit anti-human immunoglobulin G (DAKO P214).

**CIE.** CIE was performed on glass plates (5 by 7 cm) as described in detail previously (6). A new method (30) was adapted to demonstrate the specificity of antiserum to GST/TB2 fusion protein. In some plates, mouse antibody to GST/TB2 was added to the antigen well containing BCG culture fluid. The intermediate gel contained rabbit anti-mouse immunoglobulin, and, to the top gel, rabbit antibody anti-BCG 85A, anti-BCG 85B, or anti-BCG 85C was added. The first-dimension electrophoretic mobility corresponded to the horizontal bar measured as the distance from the center of the antigen well to the top of the precipitate line after projection of the stained pattern.

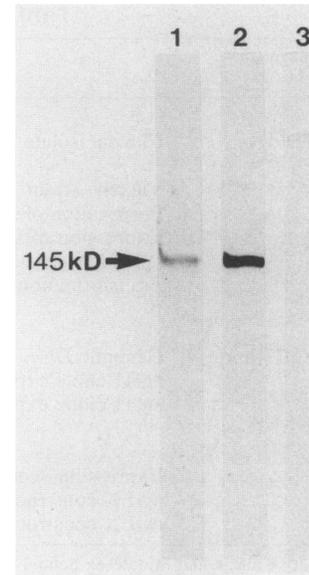


FIG. 1. Immunoblot analysis of fusion proteins expressed by recombinant clones. Anti-BCG 85 antibody was used to stain blots of extracts of *E. coli* lysogens prepared with  $\lambda$ TB1 (lane 1),  $\lambda$ TB2 (lane 2), and  $\lambda$ gt11 with no insert (lane 3).  $\beta$ -Galactosidase fusion proteins with molecular weights of  $\sim$ 145 kDa were expressed by each of the recombinant clones.

## RESULTS

**Selection and characterization of recombinant clones.** Recombinant clones were selected from an *M. tuberculosis*  $\lambda$ gt11 library screened by using a polyclonal antiserum raised against the antigen 85 complex from *M. bovis* BCG. Two clones which produced a clear signal with the antibody and which had retained *EcoRI* sites at each end of their recombinant DNA inserts were selected for detailed study. There was a difference in the intensity of antibody reactivity between the two clones, with  $\lambda$ TB2 being recognized more strongly than  $\lambda$ TB1. Western blot analysis of extracts prepared from lysogens of the two clones showed that each expressed a seroreactive  $\beta$ -galactosidase fusion protein with a molecular mass of around 145 kDa (Fig. 1).  $\lambda$ TB2 again produced a stronger signal than  $\lambda$ TB1, even though the latter clone expressed a higher level of fusion protein, visualized by ink staining (data not shown).

Analysis of DNA from the two phage clones by gel electrophoresis demonstrated the presence of 3- and 3.5-kb *EcoRI* recombinant inserts in  $\lambda$ TB1 and  $\lambda$ TB2, respectively, but detailed restriction enzyme mapping failed to show any overlapping fragments shared by  $\lambda$ TB1 and  $\lambda$ TB2 (Fig. 2). In Southern blotting experiments in which the *EcoRI* fragment from  $\lambda$ TB2 was used as the labeled probe, we were unable to demonstrate any hybridization between the two clones, indicating that TB1 and TB2 are quite distinct genes. Alignment of the restriction maps of  $\lambda$ TB1 and  $\lambda$ TB2 with those previously published for genes encoding members of the antigen 85 complex also failed to show any overlapping fragments (Fig. 2), indicating that both clones differed from the *M. tuberculosis* P32 gene and also from the  $\alpha$  antigen gene of *M. bovis* BCG, although a relationship to the latter cannot be excluded solely on the basis of restriction map comparison.

To characterize the protein products of the two genes, insert DNA from  $\lambda$ TB1 and  $\lambda$ TB2 was subcloned into the

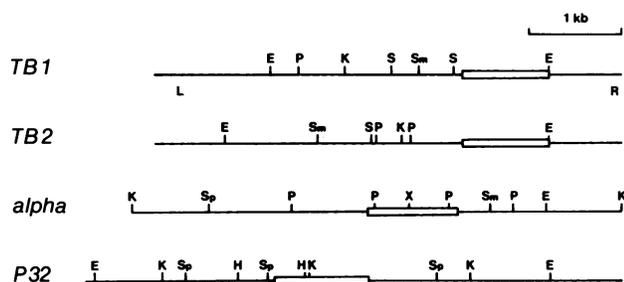


FIG. 2. Restriction maps of the *EcoRI* DNA inserts from  $\lambda$ TB1 and  $\lambda$ TB2. Regions encoding the putative structural genes are indicated by a box, as suggested by the apparent molecular weight of the fusion proteins from *lgt11* and pGEX-1 expression vectors. Orientation of lambda: L, left; R, right. Physical maps of recombinant clones encoding the  $\alpha$  antigen of *M. bovis* BCG (antigen 85B) and P32 of *M. tuberculosis* (85A) are those reported by Matsuo et al. (19) and Borremans et al. (5). Restriction enzymes: E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; S, *SalI*; Sm, *SmaI*; Sp, *SphI*; X, *XhoI*.

pGEX-1 vector, generating pGEXTB1 and pGEXTB2 expressing large amounts of GST fusion proteins. Soluble fusion proteins were obtained in each case, and these were readily purified by glutathione-agarose affinity chromatography, with yields of up to 20 mg/liter of culture. Purification of the TB1 fusion protein is illustrated in Fig. 3, with fractions monitored by ink staining for total protein (Fig. 3A) and by Western blot reactivity (Fig. 3B). The purified GST fusion protein is seen as a prominent 52-kDa band in lane 1. Monospecific antisera generated by using the purified GST/TB1 and GST/TB2 fusion proteins were used to help identify the original gene products as described below.

**$\lambda$ TB2 encodes a new member of the antigen 85 complex.** Western blot analysis with antisera raised to the GST/TB2 fusion protein revealed reactivity with a 30/31-kDa protein doublet in culture filtrates of *M. tuberculosis* (Fig. 4). Monoclonal antibody HYT27, previously shown to be specific for the antigen 85 complex (1a), reacted with GST/TB2 but showed no recognition of free GST or of the GST/TB1 fusion protein (Fig. 4). Moreover, the GST/TB2 fusion protein was recognized by FN (data not shown). Taken together, these observations strongly suggest that  $\lambda$ TB2 encodes a member of the FN-binding antigen 85 complex. The specificity of the antiserum to the GST/TB2 fusion protein was further analyzed by CIE, with the mouse antiserum incorporated in the antigen well in first-dimension gels as described previously (30). The first-dimension electrophoretic mobilities of antigen components 85A, 85B, and 85C in CIE without or with anti-GST/TB2 antiserum were measured and were the following: 16 and 15 mm for the A component, 19 mm for the B component, and 24 and 19 mm for the C component. Incorporation of the antiserum had a significant effect only on the mobility of the C component. This finding is illustrated in Fig. 5, showing that the distinct precipitate line given by antigen 85C in reference plate A was weaker and had a different position after incorporation of the antiserum in the antigen well (plate B). These results suggest that the TB2 antigen is more closely related to component C of the antigen 85 complex of *M. bovis* BCG than to component A or B.

**$\lambda$ TB1 encodes a 55-kDa FN-binding antigen.** Western blot analysis with antisera raised against the GST/TB1 fusion protein showed recognition of a 55-kDa protein band in sonic extracts of *M. tuberculosis* (Fig. 4). Together with the lack of

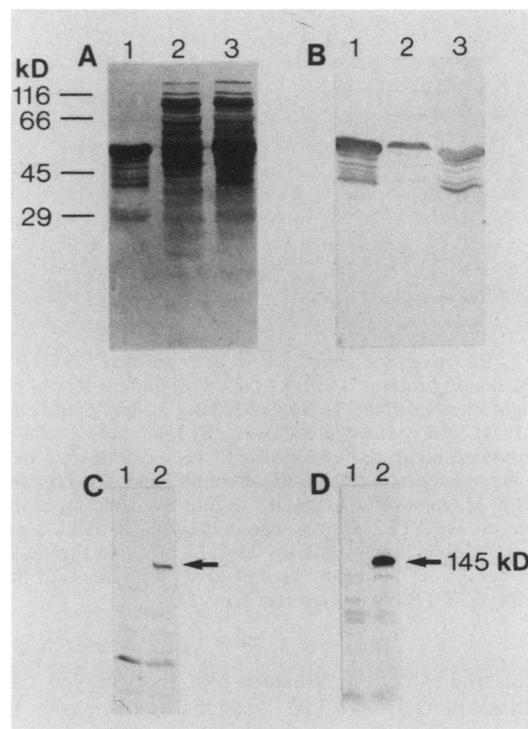


FIG. 3. Purification and serological activity of TB1 fusion proteins. (A and B) A GST fusion protein expressed by *E. coli* transformed with pGEXTB1 was purified by glutathione-agarose affinity chromatography, and fractions were analyzed by SDS-PAGE. Lane 1 shows the purified protein, with the material which passed through the column and the original bacterial extract shown in lanes 2 and 3, respectively. Panel A shows a blot stained with ink to visualize total protein, and panel B shows the same samples stained for serological activity by using a serum pool from leprosy patients. (C and D) Western blots of crude lysates of *E. coli* lysogenized with *lgt11* containing no insert DNA (lanes 1) and  $\lambda$ TB1 (lanes 2) were immunoblotted with representative sera from patients with tuberculosis (C) and with lepromatous leprosy (D).

recognition of GST/TB1 by monoclonal antibodies to the antigen 85 complex, these immunological data support the inference from genetic analysis that  $\lambda$ TB1 is not closely related to  $\lambda$ TB2. In a previous report we have shown that FN bound to proteins in the molecular weight range of 55,000 in sonicates of *M. tuberculosis* and *M. bovis* BCG (1a), and it was of interest to look for a possible relationship between the protein recognized by the anti-GST/TB1 antiserum and this second group of FN-binding proteins. Probing of blots with FN showed a specific interaction with the  $\beta$ -galactosidase fusion protein expressed by  $\lambda$ TB1, with no reactivity being seen in control extracts prepared from a *lgt11* lysogen (Fig. 6A), indicating that the cloned DNA fragment does indeed encode a polypeptide with FN-binding activity. The protein recognized by anti-GST/TB1 antiserum migrated at the same position as the major FN-binding activity in *M. tuberculosis* extracts, and pretreatment of blots with the antiserum inhibited binding of FN to the 55-kDa band (Fig. 6B). In contrast, pretreatment of immunoblots with hyperimmune mouse sera to free GST had no inhibitory effect on FN binding, again indicating identity between the 55-kDa FN-binding protein and the product of TB1.

**Recognition of TB1 by human sera.** To monitor involvement of the TB1 protein in the human antibody response to

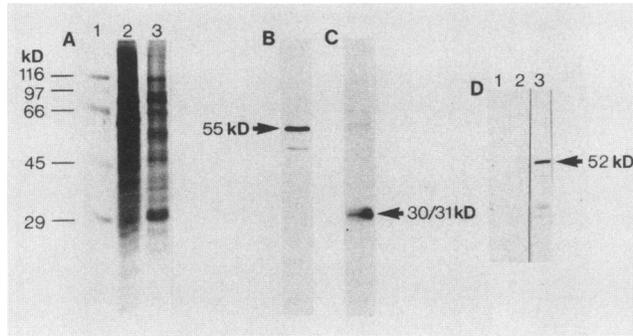


FIG. 4. Reactivity of antisera raised to purified GST/TB1 and GST/TB2 fusion proteins. (A) Ink-stained protein profiles of molecular weight markers (lane 1), sonicate (lane 2), and culture filtrate (lane 3) of *M. tuberculosis* are shown. (B) Immunoblot analysis of *M. tuberculosis* sonicate by using monospecific antiserum to GST/TB1 shows reactivity with a 55-kDa protein band. (C) Immunoblot analysis of *M. tuberculosis* culture filtrate by using monospecific antiserum to GST/TB2 shows reactivity with 30/31-kDa protein doublet. (D) Monoclonal antibody HYT27 binds to the GST/TB2 fusion protein (lane 3) but shows no reactivity with free GST (lane 1) or with the GST/TB1 fusion protein (lane 2).

mycobacterial infection, Western blot analysis was carried out with extracts from  $\lambda$ TB1 lysogens or from cells transformed with pGEXTB1. No reactivity was observed with a pool of sera from 20 healthy donors, while sera from 8 of 14 patients with pulmonary tuberculosis showed a distinct reaction with TB1 constructs but not with the free  $\beta$ -galactosidase or GST fusion partners. Positive antibody responses were also detected in sera from patients with lepromatous leprosy. Figure 3B illustrates the reactivity of a leprosy serum pool with lysates of pGEXTB1, and Fig. 3C and D show blots prepared from a  $\lambda$ TB1 lysogen probed with representative sera from tuberculosis and leprosy patients. These preliminary studies indicate that the TB1 FN-binding antigen is a prominent target of the humoral immune response to pathogenic mycobacteria.

## DISCUSSION

Some antigens of *M. tuberculosis* and *M. bovis* BCG have the interesting property of high-affinity binding to FN, a major component of the eukaryotic extracellular matrix. These antigens have been previously shown to be the secreted 30/31-kDa proteins (corresponding to the BCG 85 complex) and a group of proteins of larger molecular mass (55 to 60 kDa) found in sonicates (1a). The antigen 85 complex contains at least three components, which have been designated 85A, 85B, and 85C (32), and FN bound to all three purified antigens (1, 1a). We have used a polyclonal antiserum to the antigen 85 complex of *M. bovis* BCG to screen a DNA expression library of *M. tuberculosis* and have identified two novel genes encoding FN-binding antigens of *M. tuberculosis*.

As expected from the screening procedure used, clone  $\lambda$ TB2 encodes a member of the antigen 85 complex identified by monoclonal antibody reactivity and by the specificity of an antiserum raised against the purified recombinant product. Restriction enzyme mapping of  $\lambda$ TB2 shows that it differs from the two antigen 85 component genes previously described (85A and 85B) (5, 19), with evidence from CIE analysis indicating a relationship to the 85C component purified from *M. bovis* BCG. The apparent molecular masses

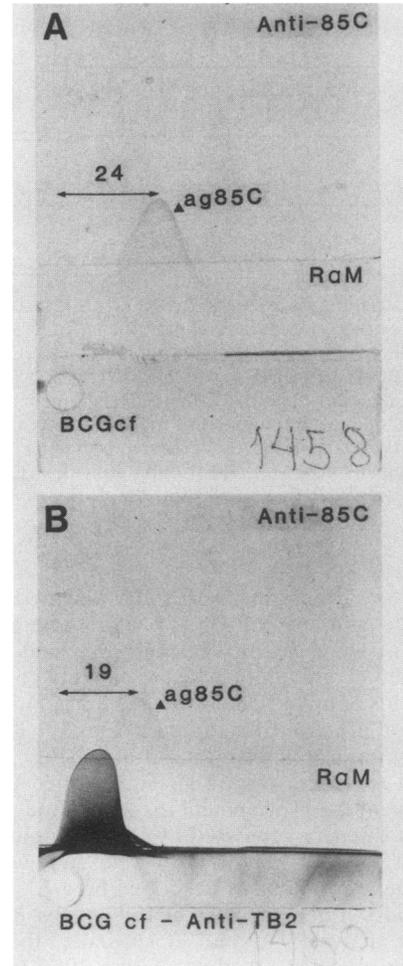


FIG. 5. Immunoelectrophoretic analysis by indirect precipitation of the antiserum to GST/TB2. (A) Reference pattern of antigen component 85C in BCG culture fluid-anti-85C antibody system. (B) Incorporation of the antiserum to GST/TB2 in the antigen well reduced the first-dimension electrophoretic mobility of the antigen.

of the  $\beta$ -galactosidase (~145 kDa) and GST (~52 kDa) fusion proteins generated from the TB2 insert suggest that most of the structural gene for the ~30-kDa mature protein product is contained within the cloned fragment. Further analysis at the nucleotide sequence level will be necessary to confirm this and to substantiate the proposed relationship to the BCG 85C component.

Unexpectedly, the second clone analyzed in detail,  $\lambda$ TB1, showed little or no relationship to the antigen 85 complex at the DNA or protein level. The TB1 antigen was identified as a 55-kDa FN-binding protein on the basis of the specificity of antisera raised against the purified recombinant fusion protein. Apart from the FN-binding activity and recognition of the TB1  $\beta$ -galactosidase fusion protein by the anti-BCG 85 antiserum, no obvious relationship is apparent between the 55-kDa antigen and the 30/31-kDa protein family. Neither the polyclonal anti-BCG 85 antiserum nor monoclonal antibody HYT27 showed significant recognition of the 55- to 60-kDa FN-binding proteins in *M. tuberculosis* extracts in Western blots (1a), and  $\lambda$ TB1 and  $\lambda$ TB2 were unrelated as assessed by Southern blotting. It is possible that some contaminating proteins were present in the antigen 85 complex used for

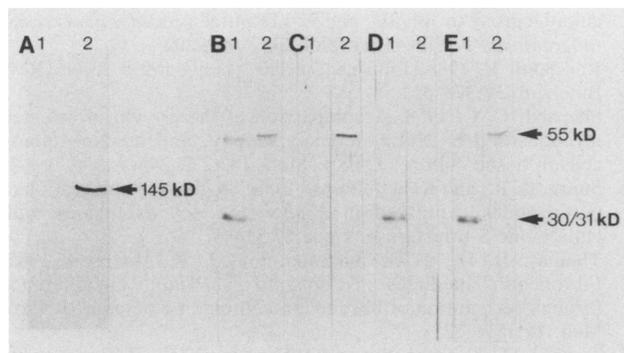


FIG. 6. Immunological relationship between the 55-kDa FN-binding protein and the antigen expressed by the recombinant phage  $\lambda$ TB1. (A) Specific binding of FN to antigen expressed by TB1. Western blots prepared from lysogens of  $\lambda$ gt11 containing no insert (lane 1) and  $\lambda$ TB1 (lane 2) were probed with FN. A single major band corresponding to the TB1/ $\beta$ -galactosidase fusion protein bound FN under these conditions. (B and C) Overlap in the recognition pattern of FN and anti-GST/TB1 antiserum. Immunoblots of culture filtrate (lanes 1) and sonicate (lanes 2) of *M. tuberculosis* were incubated with FN (B) or with mouse antiserum against GST/TB1 (C). Both reagents bound to a 55-kDa band in the sonicate, with FN also recognizing a 30/31-kDa doublet in the culture filtrate. (D and E) Specificity of inhibition of FN binding by anti-GST/TB1 antiserum. Immunoblots were reacted with FN as in panel B except that pretreatment was carried out with anti-GST/TB1 antiserum (D) or with anti-GST antiserum (E). Inhibition of FN binding to the 55-kDa protein was observed only in panel D.

immunization or, more probably, that the 55-kDa protein possesses an FN-binding epitope or some other epitope shared with components of the antigen 85 complex. However, the antibody to the GST/TB1 fusion protein was not sufficiently effective to prevent adhesion of FN to the 30/31-kDa antigens. Again, nucleotide sequence analysis will be required to address this question. From the apparent molecular weight of the fusion protein expressed by TB1 constructs, it is estimated that just over half of the structural gene is present, and the entire gene has now been isolated after screening a genomic *M. tuberculosis* EMBL3 library.

The antigen 85 complex has been identified as a major target of humoral and cell-mediated immune responses to mycobacteria (13, 21, 23), and it will be of interest to determine whether the new component represented by TB2 has any unique antigenic features distinct from those of the other family members. As judged by immunoblot analysis with TB1 fusion constructs, the 55-kDa FN-binding protein is also prominent in the immune response. Detection of a cross-reactive response to the *M. tuberculosis* antigen in sera from lepromatous leprosy patients suggests that a homologous protein may be present in *M. leprae*.

The role of FN-binding activity in the pathogenesis of mycobacterial infection remains to be elucidated. The fact that mycobacteria have multiple genes involved in the expression of large amounts of closely related 30/31-kDa proteins suggests that this family of molecules probably fulfills some essential function with regard to mycobacterial survival. Identification of the original function of these proteins, and its relation to their FN-binding affinity, may provide insights concerning their role in the host-parasite interaction. Binding to FN may be of relevance to the entry of pathogenic mycobacteria into nonprofessional phagocytic cells. FN is thought to be involved in uptake of the proto-

zoan parasite *Trypanosoma cruzi* by human phagocytes (7). *M. leprae* is found inside numerous cell types in vivo, including neurons, endothelial cells, fibroblasts, and Schwann cells, while Shepard (25) showed that several other mycobacterial species will enter HeLa, human amnion, and monkey kidney cells in vitro. In the treatment of superficial bladder tumors with intravesical *M. bovis* BCG, the intraluminal attachment of BCG appears to be mediated by FN and is necessary for the induction of antitumor activity (15). Molecular analysis of the FN-binding proteins described here may ultimately contribute, therefore, to our understanding of cancer therapy as well as of processes associated with mycobacterial infection and immunity.

#### ACKNOWLEDGMENTS

We thank Juraj Ivanyi for helpful discussions and for providing patient sera, Michael Waters and Georges Bahr for their generous gifts of sera from leprosy and tuberculosis patients, and Shilpa Ghelani for her technical assistance.

This work was supported by a grant from the World Health Organization program for vaccine development.

#### REFERENCES

1. Abou-Zeid, C. Unpublished data.
2. Abou-Zeid, C., T. L. Ratliff, H. G. Wiker, M. Harboe, J. Bennedsen, and G. A. W. Rook. 1988. Characterization of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. *Infect. Immun.* **56**:3046-3051.
3. Andersen, A. B., Z.-L. Yuan, K. Haslov, B. Vergmann, and J. Bennedsen. 1986. Interspecies reactivity of five monoclonal antibodies to *Mycobacterium tuberculosis* as examined by immunoblotting and enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **23**:446-451.
4. Beachey, E. H., and H. S. Courtney. 1987. Bacterial adherence: the attachment of group A streptococci to mucosal surfaces. *Rev. Infect. Dis.* **9**(Suppl. 5):475-481.
5. Borremans, M., L. De Wit, G. Volckaert, J. Ooms, J. De Bruyn, K. Huygen, J.-P. Van Vooren, M. Stelandre, R. Verhofstadt, and J. Content. 1989. Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of *Mycobacterium tuberculosis*. *Infect. Immun.* **57**:3123-3130.
6. Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, and M. Magnusson. 1980. The antigens of *Mycobacterium bovis* BCG, studied by crossed immunoelectrophoresis: a reference system. *Scand. J. Immunol.* **12**:249-264.
7. Cornette, J., A. Capron, and M. A. Ouassii. 1988. *Trypanosoma cruzi*: fibronectin promotes uptake of epimastigote culture forms by human neutrophils and monocytes. *Int. Arch. Allergy Appl. Immunol.* **86**:139-146.
8. Davis, R. W., D. Botstein, and J. R. Roth. 1980. *Advanced bacterial genetics: a manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. De Wit, L., A. de la Cuvellerie, J. Ooms, and J. Content. 1990. Nucleotide sequence of the 32 kDa protein gene (antigen 85A) of *Mycobacterium bovis* BCG. *Nucleic Acids Res.* **18**:3995.
10. Froman, G., L. M. Switalski, P. Speziale, and M. Hook. 1987. Isolation and characterization of a fibronectin receptor from *Staphylococcus aureus*. *J. Biol. Chem.* **262**:6564-6571.
11. Hanahan, D. 1985. Techniques for transformation of *E. coli*, p. 109-136. In D. M. Glover (ed.), *DNA cloning: a practical approach*, vol. 1. Oxford University Press, Oxford.
12. Hancock, K., and V. C. W. Tsang. 1983. India ink staining of proteins on nitrocellulose paper. *Anal. Biochem.* **133**:157-162.
13. Huygen, K., J. P. Van Vooren, M. Turneer, R. Bosmans, P. Dierckx, and J. De Bruyn. 1988. Specific lymphoproliferation, gamma-interferon production and serum immunoglobulin G

- directed against a purified 32kD mycobacterial protein antigen (P32) in patients with active tuberculosis. *Scand. J. Immunol.* **27**:187-194.
14. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in  $\lambda$ gt10 and  $\lambda$ gt11, p. 49-78. *In* D. M. Glover (ed.), *DNA cloning: a practical approach*, vol. 1. Oxford University Press, Oxford.
  15. Kavoussi, L. R., E. J. Brown, J. K. Ritchey, and T. L. Ratliff. 1990. Fibronectin-mediated Calmette-Guerin bacillus attachment to murine bladder mucosa. *J. Clin. Invest.* **85**:62-67.
  16. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
  17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  18. Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, K. Terasaka, and T. Yamada. 1990. Cloning and expression of the gene for the cross-reactive  $\alpha$  antigen of *Mycobacterium kansasii*. *Infect. Immun.* **58**:550-556.
  19. Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada. 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular  $\alpha$  antigen. *J. Bacteriol.* **170**:3847-3854.
  20. Nagai, S., H. G. Wiker, M. Harboe, and M. Kinomoto. 1991. Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. *Infect. Immun.* **59**:372-382.
  21. Pessolani, M. C. V., F. D. Rumjanek, M. A. de Melo Marques, F. S. F. de Melo, and E. N. Sarno. 1989. Serological response of patients with leprosy to a 28- to 30-kDa protein doublet from early cultures of *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* **27**:2184-2189.
  22. Ratliff, T. L., J. A. McGarr, C. Abou-Zeid, G. A. W. Rook, J. L. Stanford, J. Aslanzadeh, and E. J. Brown. 1988. Attachment of mycobacteria to fibronectin-coated surfaces. *J. Gen. Microbiol.* **134**:1307-1313.
  23. Rumschlag, H. S., T. M. Shinnick, and M. L. Cohen. 1988. Serological responses of patients with lepromatous and tuberculoid leprosy to 30-, 31-, and 32-kD antigens of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **26**:2200-2202.
  24. Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* **57**:375-413.
  25. Shepard, C. C. 1958. A comparison of the growth of selected mycobacteria in HeLa, monkey kidney, and human amnion cells in tissue culture. *J. Exp. Med.* **107**:237-245.
  26. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**:31-40.
  27. Thomas, D. D., J. B. Baseman, and J. F. Alderette. 1985. Fibronectin mediates *Treponema pallidum* cytoadherence through recognition of fibronectin cell-binding domain. *J. Exp. Med.* **161**:514-525.
  28. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
  29. Weinberg, A., and S. C. Holt. 1990. Interaction of *Treponema denticola* TD-4, GM-1, and MS25 with human gingival fibroblasts. *Infect. Immun.* **58**:1720-1729.
  30. Wiker, H. G., and M. Harboe. 1990. Integration of monoclonal antibodies in quantitative immunoelectrophoresis by indirect immunoprecipitation. *J. Immunol. Methods* **132**:127-135.
  31. Wiker, H. G., M. Harboe, and T. E. Lea. 1986. Purification and characterization of two protein antigens from the heterogenous BCG85 complex in *Mycobacterium bovis* BCG. *Int. Arch. Allergy Appl. Immunol.* **81**:298-306.
  32. Wiker, H. G., K. Sletten, S. Nagai, and M. Harboe. 1990. Evidence for three separate genes encoding the proteins of the mycobacterial antigen 85 complex. *Infect. Immun.* **58**:272-274.
  33. Young, D., T. Garbe, R. Lathigra, and C. Abou-Zeid. 1990. Protein antigens: structure, function and regulation, p. 1-35. *In* J. McFadden (ed.), *Molecular biology of the mycobacteria*. Academic Press, Ltd., London.
  34. Young, R. A., B. R. Bloom, C. M. Grosskinsky, J. Ivanyi, D. Thomas, and R. W. Davis. 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. *Proc. Natl. Acad. Sci. USA* **82**:2583-2587.