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Molecular and Immunological Characterization of *Mycobacterium tuberculosis* CFP-10, an Immunodiagnostic Antigen Missing in *Mycobacterium bovis* BCG

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In order to identify antigens that may be used in the serodiagnosis of active tuberculosis (TB), we screened a *Mycobacterium tuberculosis* genomic expression library with a pool of sera from patients diagnosed with active pulmonary TB. The sera used lacked reactivity with a recombinant form of the *M. tuberculosis* 38-kDa antigen (r38kDa), and the goal was to identify antigens that might complement r38kDa in a serodiagnostic assay. Utilizing this strategy, we identified a gene, previously designated *lhp*, which encodes a 100-amino-acid protein referred to as culture filtrate protein 10 (CFP-10). The *lhp* gene is located directly upstream of *esat-6*, within a region missing in *M. bovis* BCG. Immunoblot analysis demonstrated that CFP-10 is present in *M. tuberculosis* CFP, indicating that it is likely a secreted or shed antigen. Purified recombinant CFP-10 (rCFP-10) was shown to be capable of detecting specific antibody in a percentage of TB patients that lack reactivity with r38kDa, most notably in smear-negative cases, where sensitivity was increased from 21% for r38kDa alone to 40% with the inclusion of rCFP-10. In smear-positive patient sera, sensitivity was increased from 49% for r38kDa alone to 58% with the inclusion of rCFP-10. In addition, rCFP-10 was shown to be a potent T-cell antigen, eliciting proliferative responses and gamma interferon production from peripheral blood mononuclear cells in 70% of purified protein derivative-positive individuals without evident disease. The responses to this antigen argue for the inclusion of rCFP-10 in a polyvalent serodiagnostic test for detection of active TB infection. rCFP-10 could also contribute to the development of a recombinant T-cell diagnostic test capable of detecting exposure to *M. tuberculosis*.

Mycobacterium tuberculosis, the causative agent of pulmonary tuberculosis (TB), has infected approximately one-third of the world population and is projected to result in 3.5 million annual deaths by the end of the year 2000 (18). The bacterium is spread primarily through aerosolized infectious particles generated from coughing and sneezing by individuals with TB. Due to the primary route of transmission, early diagnosis of TB is essential in limiting the spread of *M. tuberculosis* within a human population. An optimal diagnostic test for TB would be able to detect early disease with high sensitivity and specificity, would yield results with rapidity, would be inexpensive, and would cause little or no patient discomfort. Currently available methods for the diagnosis of TB, including microscopic examination of sputum smears (24), culturing of sputum samples (8), PCR-based detection systems (8), detection of lipoarabinomannan in sera (19), chest X-ray, and the Mantoux test, all fail to satisfy at least one of the above requirements. An attractive methodology that continues to be explored is the detection of *M. tuberculosis*-specific antibody in patient sera, since a serodiagnostic test could potentially satisfy all of the requirements for an optimal diagnostic test for TB.

Although numerous *M. tuberculosis* antigens capable of generating specific antibody titers in TB patients have been identified (16, 24), no single antigen appears to be ideal for serodiagnostic use. One of the best diagnostic markers isolated thus far, the 38-kDa antigen (3, 6), has shown some potential for use in a single-antigen diagnostic test. Studies using the antigen in an enzyme-linked immunosorbent assay (ELISA) format detected up to 85% of smear-positive cases (5, 10, 13). However, as a single antigen, the 38-kDa antigen still lacks sufficient sensitivity to create an optimal serodiagnostic test, especially for smear-negative individuals, where sensitivity is considerably lower (13).

We were interested in determining if there are additional *M. tuberculosis* antigens that would complement a recombinant 38-kDa antigen (r38kDa) for serodiagnostic testing to detect active TB. Previous work of our own and by others utilizing expression screening with TB patient sera on recombinant *M. tuberculosis* libraries demonstrated the effectiveness of this approach in recovering antigens recognized by patient sera (2, 9; M. J. Lodes, D. C. Dillon, R. L. Houghton, R. Raodoh, C. Day, D. R. Benson, L. D. Reynolds, P. D. McNeill, and S. G. Reed, unpublished data). In order to isolate antigens that might complement the r38kDa antigen, we used expression screening of a genomic *M. tuberculosis* library with a pool of TB patient sera determined previously to lack reactivity with the r38kDa antigen. This approach is supported by a previous study that found that the antibody response to *M. tuberculosis* antigens in different infected individuals is heterogeneous (16). Additional support is found in the preferential recognition of an 81-kDa *M. tuberculosis* antigen in human immunodeficiency virus (HIV)-infected TB patients (11, 15).

Utilizing this approach, we have isolated a gene we initially referred to as *mtb11*, which encodes a 100-amino-acid protein, Mtb11 (1). The same gene has recently been designated *lhp*

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CFP-10              MAEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQAAVVRFQE  60
rCFP-10             MAEMK.......................................................  60
rΔCFP-10 MHHHHHHPGCRNSARE..................................................  71

CFP-10          AANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQMGF                      100
rCFP-10         ....................................HHHHHH                     106
rΔCFP-10        ....................................                          111
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FIG. 1. Amino acid sequences of native CFP-10, rCFP-10, and rΔCFP-10. Residues that are identical in all three proteins are represented by dots.

(L45 homologous protein), and the encoded protein has been designated culture filtrate protein 10 (CFP-10) (5) and MTSA-10 (*M. tuberculosis*-specific antigen 10) by another group (7). Herein, we demonstrate that this protein is recognized by a subset of TB patient sera and also by peripheral blood mononuclear cells (PBMC) from a subset of healthy, purified protein derivative-positive (PPD^+) individuals. These results suggest potential roles for this antigen both in complementing the r38kDa antigen in the serodiagnosis of active TB and as part of a polyvalent recombinant T-cell diagnostic test to detect *M. tuberculosis* exposure.

MATERIALS AND METHODS

Bacterial strains. *M. tuberculosis* strains H37Rv, H37Ra, and Erdman were gifts from the Seattle VA Hospital; the "C" strain was a gift from Lee Riley, University of California, Berkeley; and *M. bovis* BCG and *M. leprae* (Pasteur) were obtained from Genesis Corp., Auckland, New Zealand. The following other species of mycobacteria were obtained from the American Type Culture Collection (ATCC; Manassas, Va.): *M. vaccae* (ATCC 15483), *M. avium* subsp. *avium* (ATCC 35718), *M. chelonae* (ATCC 14472), *M. fortuitum* (ATCC 6841), *M. gordonae* (ATCC 14470), *M. scrofulaceum* (ATCC 19981), and *M. smegmatis* (ATCC 19420). The *M. tuberculosis* H37Rv CFPs and membrane fraction were provided by John Belisle, Colorado State University, Fort Collins.

Study population. Serum samples were obtained from both male and female individuals (>18 years of age) who had pulmonary TB alone prior to treatment (culture and/or acid-fast bacillus smear positive and negative). These were obtained from the Federal University of Bahia, Salvador, Brazil. To evaluate the specificity of the recombinant CFP-10 (rCFP-10) antigen, we obtained sera from individuals who were PPD^+ (>10 mm) (culture, clinically, and radiographically negative for TB) and from PPD^- individuals (King County TB Clinic, Seattle, Wash.). Additional healthy blood donors (United States) were obtained from Boston Biomedica, West Bridgewater, Mass.). PBMC were obtained from either the blood or apheresis product of healthy PPD^+ or PPD^- individuals by density centrifugation over Ficoll. None of the PPD^+ donors included in the PBMC assay had a history of BCG immunization. All of the sera and cells used came from individuals who were HIV negative.

Isolation of *M. tuberculosis* clones. *M. tuberculosis* H37Ra genomic DNA was isolated and sheared by sonication to a size range of 1 to 4 kb. *M. tuberculosis* H37Rv genomic DNA was partially digested with *Sau*3A1. Libraries were constructed in Lambda ZapII (Stratagene, La Jolla, Calif.) using *Eco*RI adapters. Expression screening was performed using a pool of eight patient sera preadsorbed with *Escherichia coli* (20). These eight serum samples were demonstrated to lack reactivity with the r38kDa antigen by ELISA. Additionally, seven of the eight samples came from smear-negative TB patients.

Cloning of the full-length *mtb11* (*lhp*) gene was accomplished by isolating the 5′ portion of the RaCl-1 insert, random labeling it with [^{32}P]dCTP, and screening approximately 75,000 plaques as previously described (20) using the H37Rv genomic library.

Expression of recombinant *M. tuberculosis* antigens. The 1.4-kb insert within the original RaCl-1 clone was subcloned by restriction into a pET17b vector, modified by the addition of residues encoding a six-histidine tag following the ATG start codon within the *Nde*I site, followed by the inclusion of a portion of the pBSK polylinker (region from *Sma*I to *Xho*I). The resulting clone was referred to as pETΔlhp, and the resulting recombinant protein was named rΔCFP-10 and contained the amino acid sequence MHHHHHHPGCRNSARE, followed by the 95 C-terminal residues of CFP-10 (Fig. 1).

The full-length *lhp* gene contained within the RvCl-1 clone was engineered for expression by PCR utilizing a 5′ primer which contained an *Nde*I site (which included the ATG start codon of *lhp*) and a 3′ primer which contained residues encoding the C-terminal residues of the *lhp* gene, a six-histidine tag, a termination codon, and a *Hin*dIII site. The amplified product was digested with *Nde*I and *Hin*dIII and ligated into pET17b. This clone was referred to as pETlhp, and the recombinant protein encoded was rCFP-10. All DNA manipulations were confirmed by DNA sequencing to eliminate the possibility of the introduction of mutations by restriction, ligation, and PCR.

Expression and purification of rΔCFP-10 and rCFP-10 were performed as follows. Pellets from induced *E. coli* BL-21(pLysE) containing the pETΔlhp and pETlhp constructs were lysed, and the recombinant proteins were recovered in the soluble fraction. The initial purification of rΔCFP-10 was performed by binding the sample with Ni-nitrilotriacetic acid (Ni-NTA) resin under nondenaturing conditions (20 mM Tris-HCl [pH 8], 100 mM NaCl), washing with 20 mM Tris-HCl [pH 8]–100 mM NaCl–10 mM imidazole, and elution in a column with increasing concentrations of imidazole (10 to 100 mM). Fractions containing protein were combined and dialyzed against 10 mM Tris-HCl (pH 7.4). This initial purification method resulted in the copurification of approximately equal amounts of recombinant ESAT-6 (rESAT-6) protein. These recombinant TB proteins were separated on a Vydac C_{18} (218TP5115) column (1 by 150 mm) by reverse-phase high-pressure liquid chromatography (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). The proteins were separated on a linear gradient of 0 to 100% buffer B in 50 min. The two major peaks eluted at 39 and 47 min on the chromatogram. The peak fractions were loaded directly into the Procise 494 protein Sequencer (Perkin-Elmer/Applied Biosystems). The amino-terminal protein sequence identified the two peaks as rΔCFP-10 and rESAT-6, respectively. The *esat-6* gene is located just downstream of *lhp* and is present in the pETΔlhp clone, which contains a 1.4-kb insert. This allowed the coexpression of rΔCFP-10 and rESAT-6 in the induced *E. coli* and subsequent copurification of the proteins on Ni-NTA resin, presumably due to an interaction between these two proteins.

Later purifications of rΔCFP-10 and rCFP-10 were performed as described above, except that they were performed under denaturing conditions, with the inclusion of 8 M urea during binding, washing, and elution on Ni-NTA resin. In all cases, the purity of the recombinant protein was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining, and N-terminal sequencing using traditional Edman chemistry with a Procise 494 protein Sequencer (Perkin-Elmer/Applied Biosystems). The endotoxin level was determined to be less than 100 endotoxin units/mg by *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.).

Expression and purification of r38kDa were performed as described previously (11).

Molecular analysis of *M. tuberculosis* clones. DNA was prepared by following the manufacturers' (Qiagen, Chatsworth, Calif., and Promega, Madison, Wis.) protocols. DNA sequencing was performed by automated sequencer (model 373; Perkin-Elmer/Applied Biosystems). DNA sequences and deduced amino acid sequences were used in database searches (GenBank DNA and protein databases).

Genomic DNAs from mycobacterial strains were digested with *Pst*I, separated by agarose gel electrophoresis, and blotted on Nytran (Schleicher & Schuell, Keene, N.H.). The *lhp* gene was labeled with [^{32}P]dCTP by random oligonucleotide primers (Boehringer Mannheim, Indianapolis, Ind.) and used as a probe. Hybridization was performed at 65°C in 0.2 M Na H_2PO_4–3.6 M NaCl–0.2 M EDTA overnight and washed to a stringency of 0.075 M NaCl–0.0075 M sodium citrate (pH 7.0)–0.5% SDS at the temperature of hybridization.

Immunoblot analysis. Antiserum to rΔCFP-10 was raised using adult New Zealand White rabbits (R & R Rabbitry, Stanwood, Wash.) as described previously (9).

M. tuberculosis H37Rv lysate, CFP, and purified rCFP-10 were subjected to SDS–12% PAGE and transferred to nitrocellulose. Filters were blocked with phosphate-buffered saline (PBS; pH 7.4) containing 5% nonfat milk at 4°C overnight, washed three times in PBS–0.1% Tween 20 (PBS-T), and incubated for 1 h in rabbit serum (diluted 1:250 in PBS-T) on a rocker at room temperature. Filters were washed three times with PBS-T, and bound antibody was detected with 10^5 cpm of ^{125}I-labeled protein A per ml, followed by autoradiography.

Serological analysis of rCFP-10. ELISAs were performed with 96-well microtiter plates (Corning Costar, Cambridge, Mass.) which were coated with CFP-10 and 38-kDa antigen (200 ng/well) and incubated overnight at 4°C. ELISAs were performed as described previously (11). The cutoff for the assays was the mean of the negative population plus 3 standard deviations (SD) of the mean.

Proliferation and cytokine production assays. PBMC were cultured in 96-well round-bottom plates (Corning Costar) at 2×10^5 cells/well in a volume of 200 μl. Antigens were tested in triplicate at 2 to 10 μg/ml. The culture medium consisted of RPMI medium with 10% pooled human serum and gentamicin at 50 μg/ml. After 5 days of culture at 37°C in 5% CO_2, 50 μl of culture supernatant was carefully aspirated for determination of gamma interferon (IFN-γ) levels and the plates were pulsed with 1 μCi of tritiated thymidine per well. After culture for a further 18 h, cells were harvested and tritium uptake was determined using a gas

scintillation counter. IFN-γ levels in culture supernatants were determined by ELISA as previously described (21). Proliferation results were considered positive if the stimulation index (SI) compared to that of a no-antigen control was 5 or greater. This cutoff was selected based on previous work involving approximately 20 purified recombinant *M. tuberculosis* antigens. It was found that PPD$^-$ donor PBMC responses to these antigens rarely exceeded an SI of 5 (data not shown). Additionally, when the mean SI plus 3 SD was calculated for this group of samples (healthy, PPD$^-$ PBMC) for these various antigens (including rCFP-10), a value of approximately 5 was obtained (data not shown).

RESULTS

Isolation of *lhp*. A pool of sera from eight individuals with active or recently treated pulmonary TB was used in expression screening of an *M. tuberculosis* H37Ra genomic library. These eight sera had previously been shown to lack reactivity to purified *M. tuberculosis* r38kDa by ELISA (data not shown). Screening of 40,000 plaques led to the isolation of a single reactive clone, termed RaCl-1, containing an insert of approximately 1.4 kb. Partial DNA sequence analysis was performed on the RaCl-1 clone, which predicted an encoded 95-amino-acid open reading frame in frame with the N-terminal 4-kDa β-galactosidase, creating a fusion protein of approximately 14 kDa. The RaCl-1 clone was re-engineered to remove most of the 5′ *lacZ* sequence and add a six-histidine tag for use in purification of the recombinant protein (see Materials and Methods). The re-engineered clone was designated pETΔlhp, and the protein was designated rΔCFP-10 (Fig. 1).

The 5′ portion of the RaCl-1 insert was used as a probe to recover the full-length gene from an *M. tuberculosis* H37Rv genomic library. Six clones were recovered, and subsequent DNA sequencing determined that one (RvCl-1) contained the full-length gene which included an additional 5 N-terminal amino acids not present in the original fusion, resulting in a predicted protein of 100 amino acids (Fig. 1) with a mass of 10,794 Da. No differences at the DNA level between the RaCl-1 and RvCl-1 clones were identified within the coding region of this gene (data not shown).

We initially referred to this gene as *mtb11* and the encoded protein as Mtb11, based on the predicted mass of the protein (1). Subsequent DNA database searches with this sequence revealed 100% identity with an *M. bovis* sequence which was previously demonstrated to be within a region missing in *M. bovis* BCG (17). In addition, identity to the *M. tuberculosis lhp* gene, located just upstream of *esat-6*, was found. A search performed with the predicted amino acid sequence showed identity to the putative open reading frame encoded by the *lhp* gene (accession no. CAA17966), referred to in the database as CFP-10. The *lhp* gene 3′ end is located just 34 bp upstream of the *esat-6* gene, and both genes are oriented in the same direction. Additionally, evidence has been presented that the two genes are part of an *M. tuberculosis* operon and that both proteins are secreted (4, 23). The protein database searches did not reveal significant homology with any proteins with known function but did show some relatedness (40% identity) to a predicted 100-amino-acid *M. leprae* protein (accession no. CAA75210) and low-level relatedness to ESAT-6 and TB10.4 (22). Hydropathy analysis (14) of the CFP-10 amino acid sequence did not indicate the presence of any extended hydrophobic regions that might serve as transmembrane domains (data not shown).

Genomic DNAs from a number of mycobacterial species were analyzed by Southern blotting using the *lhp* gene as a probe. The results (Fig. 2) indicate that the gene is present in single copy and is conserved in two *M. tuberculosis* clinical strains but not well conserved in any of the other mycobacterial species tested, including *M. bovis* BCG.

The re-engineered clone, pETΔlhp, comprised the entire

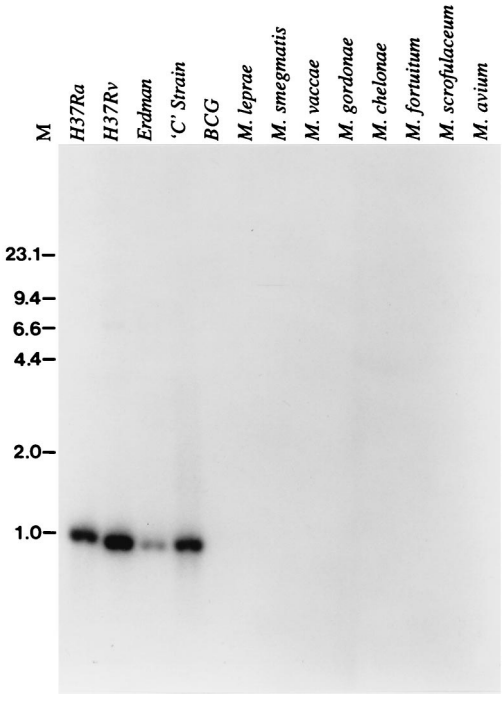

FIG. 2. Southern blot analysis of the *lhp* gene. Genomic DNAs (2.5 μg) from mycobacterial strains were digested with *Pst*I, separated by agarose gel electrophoresis, and blotted onto Nytran. The *lhp* gene was labeled with [^{32}P]dCTP by random oligonucleotide primers and used as a probe. Molecular size markers (M) in kilobases are shown.

1.4-kb insert originally recovered, which included the entire *esat-6* gene region located immediately downstream of the *lhp* gene (see Materials and Methods). An unanticipated consequence of the expression and purification of rΔCFP-10 using Ni-NTA resin (Qiagen) under nondenaturing conditions was the coexpression and copurification of recombinant ESAT-6 in amounts roughly equivalent to those of rΔCFP-10. Under the purification conditions used, the coisolation of rESAT-6 had to occur through interaction of the rESAT-6 protein with either rΔCFP-10 or directly with the Ni-NTA resin. Since the *esat-6* gene in this construct was not engineered to contain a six-histidine tag, the interaction of rESAT-6 with rΔCFP-10 is the more likely explanation. This finding is consistent with earlier work indicating that these genes are coordinately expressed (4) but also indicates that the two proteins may interact directly.

To characterize native *M. tuberculosis* CFP-10, rabbit antiserum to purified rΔCFP-10 was raised. This antiserum was used in an immunoblot assay of the *M. tuberculosis* lysate, CFP, and membrane fraction. The results (Fig. 3) indicate the presence of a reactive protein with an approximate mass of 11 kDa in the lysate, CFP, and membrane fraction.

Immunological responses to rCFP-10. The full-length *lhp* gene was reengineered for expression, removing all flanking sequences and adding a C-terminal six-histidine tag (Fig. 1). The resulting recombinant antigen, referred to as rCFP-10, was expressed and purified (Fig. 4) and was used to assess human antibody and T-cell responses.

Sera from individuals with TB were assessed for specific antibody reactivity with r38kDa and rCFP-10 in an ELISA format (Table 1). These included sera from both smear-positive and smear-negative TB patients. The control sera used were from healthy PPD$^+$ and PPD$^-$ individuals. Serological reactivity to rCFP-10 was observed in 28% (69 of 250) of

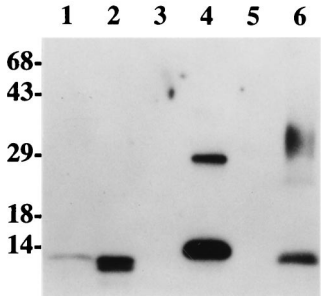

FIG. 3. Characterization of native CFP-10. *M. tuberculosis* H37Rv lysate (2.5 μg; lane 1), 2.5 μg of CFP (lane 2), 2.5 μg of the cytoplasmic fraction (lane 3), 50 ng of rCFP-10 (lane 4), no sample (lane 5), and 2.5 μg of the membrane fraction (lane 6) were subjected to SDS-PAGE, transferred to nitrocellulose, and reacted with rabbit antiserum generated against rΔCFP-10. Molecular masses are shown in kilodaltons.

smear-positive TB patient sera and in 25% (13 of 52) of smear-negative TB patient sera. Reactivity was generally not observed in sera from either healthy PPD^+ or healthy PPD^- individuals, with an overall specificity of approximately 97% (83 of 86).

Moreover, rCFP-10 was able to complement r38kDa in the detection of TB. In smear-positive TB patient sera, detection increased from 49% (122 of 250) with r38kDa alone to 58% (146 of 250) with both r38kDa and rCFP-10. More significant complementation was observed in smear-negative patient sera, where detection increased from 21% (11 of 52) with r38kDa alone to 40% (21 of 52) detected with both r38kDa and rCFP-10. The additional sensitivity provided by the addition of rCFP-10 to r38kDa did not result in significant specificity problems, with no additional rCFP-10-positive, PPD^+ sera and one additional rCFP-10-positive, PPD^- control serum. Thus, the specificity of r38kDa alone was 93% (80 of 86) and that of the combination of r38 kDa and rCFP-10 was 92% (79 of 86). In total, there were three sera that yielded false-positive results with rCFP-10 (from two PPD^+ individuals and one PPD^- individual). The latter was at the edge of the cutoff of the assay and was of U.S. origin. Of the remaining two (PPD^+) individuals, the one with the highest ELISA reactivity was of U.S. origin, had been in contact with an active case, was a potential converter, and was being placed on therapy. The remaining false positive person for CFP-10 was from South Vietnam.

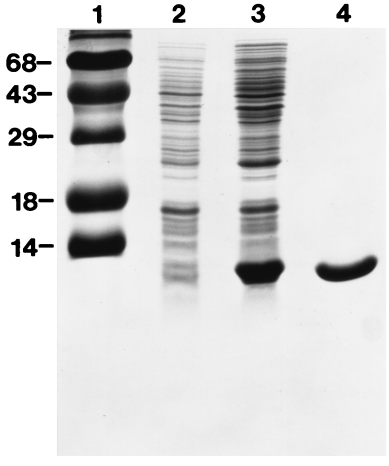

FIG. 4. Purification of rCFP-10. Expression and purification of rCFP-10 are shown with uninduced (lane 2) and induced (lane 3) *E. coli* lysates and 5 μg of purified protein (lane 4). Molecular masses are shown in kilodaltons (lane 1).

T-cell responses to rCFP-10 were also evaluated. PBMC from 20 healthy PPD^+ donors and 20 healthy PPD^- donors were analyzed for proliferation and production of IFN-γ in response to CFP or purified rCFP-10. Using an SI cutoff of 5 (see Materials and Methods), purified rCFP-10 elicited proliferative responses in 70% (14 of 20) PPD^+ donors but no response was observed in the 20 PPD^- donors (Table 2). This contrasted with CFP, which elicited proliferative responses from the PBMC of 95% (19 of 20) of healthy PPD^+ individuals but also elicited responses from 45% (9 of 20) of healthy PPD^- individuals. The difference in mean SI between the PPD^+ group and the PPD^- group was 17-fold for rCFP-10, while only a 7-fold difference was observed for CFP. Analysis of the IFN-γ production for these same samples yielded responses similar to the proliferation results (data not shown).

Finally, for those samples in which both PBMC and serum were available, we compared T-cell responses and antibody responses to rCFP-10 (Table 3). The data show clearly that regardless of the presence and extent of the T-cell response to rCFP-10 observed in PBMC in a subset of the healthy PPD^+ donors (eight of nine with an SI of greater than 5), no detectable antibody response was present. No positive rCFP-10-specific T-cell response or antibody reactivity was observed for any of the PPD^- individuals.

DISCUSSION

A significant difficulty in the development of a serodiagnostic assay for detection of TB has been the identification of an antigen, or a collection of antigens, that yields the desired sensitivity and specificity. The *M. tuberculosis* 38-kDa antigen is undoubtedly the best single serodiagnostic antigen to have been identified thus far, but it still has sensitivity limitations, especially in smear-negative individuals (13). However, if additional *M. tuberculosis* antigens are to be used to complement the 38-kDa antigen to yield increased sensitivity in a serodiagnostic assay, it cannot occur at the price of specificity. We have demonstrated that both the serological and T-cell responses to rCFP-10 antigen are highly specific. It is possible that the specificity observed with rCFP-10 is due, at least in part, to the fact that the gene encoding this antigen is not highly conserved in other mycobacterial species and is missing in *M. bovis* BCG. Consequently, cross-reactive responses are not likely to occur from previous exposure to other mycobacterial species or through immunization with *M. bovis* BCG. We have observed that BCG-immunized individuals do not have increased rCFP-10-specific antibody responses (data not shown). Although we have not tested sera or PBMC from individuals infected with other mycobacterial species, such as *M. avium*, the lack of conservation of this gene in these mycobacteria predicts no rCFP-10-specific responses from the sera or PBMC of these patients.

Although the TB patient group used in these serological studies had a singular geographic origin (Brazil), additional studies that are ongoing show similar serological responses to rCFP-10 from TB patients from other geographical areas (data not shown). Additionally, because the patient cohort was located in an area where TB is endemic, it is unlikely that the disease in these patients was caused by nontuberculous mycobacteria. As a stand-alone antigen, rCFP-10 would be a poor serodiagnostic agent, detecting only 28% of smear-positive TB patients and 25% of smear-negative TB patients. However, we found that rCFP-10 does have potential as a supplemental antigen to r38kDa, most notably in smear-negative TB patients, where sensitivity was increased from 21 to 40% without

TABLE 1. Serological reactivity of rCFP-10 and r38kDa in smear-positive and smear-negative TB patients and healthy PPD$^+$ and PPD$^-$ individuals

Status	Origin	Smear result	No. of persons	No. of samples positive by ELISA[a] for:		
				rCFP-10[b]	r38kDa	r38kDa + rCFP-10
TB	Brazil	+	250	69	122	146
		−	52	13	11	21
Healthy, PPD$^+$	Africa		3	0	0	0
	Europe, Asia		6	0	0	0
	Southeast Asia		7	1	2	2
	U.S., Latin America		41	1	3	3
Total PPD$^+$			57	2	5	5
Healthy PPD$^-$	Africa		1	0	0	0
	Europe, Asia		3	0	0	0
	Southeast Asia		3	0	0	0
	U.S., Latin America		22	1	1	2
Total PPD$^-$			29	1	1	2

[a] Positive values were greater than the mean plus 3 SD of the sera of healthy PPD$^-$ persons.
[b] Positive ELISA values for rCFP-10 and TB patient sera ranged from 0.2 to >3.0.

a significant reduction in test specificity. Although these two antigens together are clearly not sufficient, lacking the necessary sensitivity of an optimal diagnostic agent to detect active TB, these results do argue for the possibility that a multiantigen serodiagnostic test for TB with high sensitivity can be achieved while maintaining a high level of specificity. Current evaluation of an additional *M. tuberculosis* antigen, MTB48, that can further complement the combination of r38kDa and rCFP-10 in the serodiagnosis of TB also supports this multiantigen diagnostic approach (Lodes et al., unpublished). Additional evidence that specific antigens are capable of assisting in the serodiagnosis of specific subgroups of active TB has been found in a recent study demonstrating the use of Mtb81 in the detection of active TB in patients coinfected with HIV (11). Collectively, the data indicate that a set of antigens will likely be needed to achieve a serodiagnostic test with the desired sensitivity. However, each antigen component must contribute to increasing the sensitivity of the serodiagnostic test and maintaining a high degree of specificity for inclusion in a multiantigen serodiagnostic test for active TB. The ability of rCFP-10 to satisfy both of these criteria qualifies it as a supplemental antigen to r38kDa.

An additional diagnostic tool would be one that could detect exposure of individuals to *M. tuberculosis*. The current diagnostic agent PPD, although both sensitive and inexpensive, has specificity problems, yielding positive results in some individuals infected with other mycobacterial strains or in BCG-immunized individuals (12). A superior diagnostic may be found in a collection of recombinant *M. tuberculosis* antigens that yield specific T-cell responses, such as rCFP-10, that could be utilized in a skin test or an in vitro T-cell diagnostic assay. In fact, rCFP-10 appears to be able to contribute more robustly as a T-cell diagnostic antigen, with proliferative responses from PBMC seen in 70% of PPD$^+$ donors and no positive responses observed in the PPD$^-$ donors tested. Consistent with the role of CFP-10 as a potential T-cell diagnostic antigen, a recent study with this antigen has demonstrated that it is capable of eliciting delayed-type hypersensitivity in *M. tuberculosis*-infected guinea pigs but not in *M. bovis* BCG-infected or *M. avium*-infected guinea pigs (7). Additionally, it has been re-

TABLE 2. PBMC responses to rCFP-10 and CFP from healthy PPD$^+$ and PPD$^-$ donors[a]

Status	rCFP-10		CFP	
	No. positive/total (%)	Mean SI	No. positive/total (%)	Mean SI
PPD$^+$	14/20 (70)	31.4	19/20 (95)	69.1
PPD$^-$	0/20 (<5)	1.9	9/20 (45)	9.6

[a] PBMC from donors were stimulated with rCFP-10 or CFP at 10 μg/ml. Samples were considered positive if the SI was >5.

TABLE 3. PBMC response[a] and antibody reactivity[b] to rCFP-10 of healthy PPD$^+$ and PPD$^-$ donors

Donor	rCFP-10 PBMC SI[a]	rCFP-10 ELISA OD[b]
PPD$^+$		
D7	96.5	0.102
D27	15.1	0.050
D62	34.6	0.088
D103	31.5	0.010
D131	3.0	0.020
D152	57.6	0.080
D160	60.3	0.069
D184	99.7	0.034
D366	5.8	0.061
PPD$^-$		
D16	1.0	0.022
D17	0.3	0.063
D44	0.6	0.129
D65	0.6	0.042
D101	0.7	0.051
D109	0.6	0.052
D140	1.4	0.062
D149	0.6	0.061
D150	3.6	0.110
D206	1.9	0.108
D231	0.4	0.067
D244	0.6	0.081

[a] PBMC from donors were stimulated with rCFP-10 at 10 μg/ml.
[b] Positive ELISA value set at mean value of PPD$^-$ sera + 3 SD = 0.2.

cently demonstrated that PBMC from TB patients generated IFN-γ production in response to recombinant CFP-10 (22). These data, along with the data presented here on responses by healthy PPD^+ donors, support the use of rCFP-10 as a component of a T-cell diagnostic antigen for the detection of exposure to *M. tuberculosis*.

One unexpected result of the characterization of CFP-10 was the discovery that it may interact with ESAT-6. Supporting this interaction are the adjacent location of these two genes, the evidence that they are cotranscribed (4), and the finding presented here describing the copurification of rESAT-6 with rΔCFP-10 when expressed in *E. coli*. Lastly, there is also some relatedness between ESAT-6 and CFP-10 at the amino acid level (22). Collectively, these data encourage speculation that is there not only coordinated expression but also some direct interaction between CFP-10 and ESAT-6 in *M. tuberculosis*. Like ESAT-6, CFP-10 can be found in CFP from *M. tuberculosis* in vitro cultures. However, the level of CFP-10 expression and protein localization in active and dormant *M. tuberculosis* in a human infection is not known at this time.

It has been well established that the generation of substantive antibody responses to protein antigens is dependent on the existence of T-cell epitopes within the antigen recognized by helper T lymphocytes. Thus, specific serological responses elicited during pulmonary TB provide a mechanism by which to identify *M. tuberculosis* T-cell antigens (9). These same antigens, while generating a detectable humoral response in susceptible individuals, may also be a target of cellular responses in the more frequent outcome of *M. tuberculosis* infection, i.e., that of acquired protective immunity. Support exists for these dual responses to the *M. tuberculosis* 38-kDa antigen (25), and the data presented herein support the idea that CFP-10 is one such antigen. It is recognized by the immune systems of a substantial percentage of individuals infected with *M. tuberculosis*, both those with active disease and those that have developed protective responses. The data demonstrate that the immunological responses to rCFP-10 by protected and susceptible individuals are qualitatively different at the time of measurement. The dual responses to this antigen afford it potential utility both as a component of a serodiagnostic test for active TB and as a component of an improved skin test antigen to detect exposure to *M. tuberculosis*.

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