Chapter 2:

MATERIALS AND METHODS
2.1. MATERIALS

The expression vectors pET22b and pET28b used for cloning of esxA and esxB genes were obtained from Novagen (Darmstadt, Germany). The vector pQE60 was from Qiagen (Hilden, Germany). Oligonucleotides for gene isolation were from Sigma-Genosys (Bangalore, Karnataka, India). Gene amplification from *M. tuberculosis* H37Rv genomic DNA was performed using high fidelity PCR enzyme mix and dNTPs from Fermentas (Genetix, New Delhi, India). Restriction Endonucleases, T4 DNA Ligase and DNA size markers were from New England Biolabs (Beverly, MA, USA). Plasmid Miniprep kit, the Maxiprep kit, and the Gel extraction kit used for plasmid preparations and DNA purification processes, respectively, were obtained from Qiagen (Genetix, New Delhi, India). The pGEM-T T/A cloning kit was from Promega (Madison, USA). Tryptone and yeast extract powder used for preparation of Luria-Bertani media, and agar powder were from HiMedia, India. IPTG was obtained from Calbiochem (USA). $^{15}$N-ammonium sulfate, $^{13}$C-D-glucose, $^2$H$_7$,$^{13}$C-D-glucose, CDN100-Bioexpress and D$_2$O (99.92%) used for labeling of the proteins were obtained from Cambridge Isotope Limited, Inc. (Andover, MA, USA). The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and protease inhibitor cocktail (containing AEBSF, pepstatin A, E-64, bestatin, and phosphoramidon) were from Sigma (New Delhi, India). Nickel-nitrilotriacetic Acid (Ni-NTA) Superflow metal-affinity chromatography matrix was obtained from Qiagen (Genetix, New Delhi, India). Amicon YM-3 (MWCO 3 kDa) ultrafiltration membrane used for concentrating purified proteins was from Millipore Pvt. Ltd. (Bangalore, India). L-1-Tosylamide-2-phenylchloromethyl ketone (TPCK) treated trypsin (from bovine pancreas) used for proteolysis experiment was from Sigma (New Delhi, India). RPMI 1640 and FBS were obtained from Gibco BRL (USA). [3H]thymidine was from BARC (Mumbai, India). The interleukins IFN-γ and TNF-α were quantified using ELISA kit from BD Biosciences (Mumbai, Maharashtra, India). Rests of the chemical reagents were obtained from Sigma (New Delhi, India) and SRL (Mumbai, Maharashtra, India).
2.2. METHODS

2.2.1. Cloning of the genes CFP-10 (Rv3874) and ESAT-6 (Rv3875) from *M. tuberculosis* H37Rv in *E. coli* expression vectors

2.2.1.1. Preparation of genomic DNA of *M. tuberculosis* H37Rv

Genomic DNA of *Mycobacterium tuberculosis* H37Rv was prepared by the method described by Kremer et al. (2005). Briefly, a volume of 100 μl of packed cells of *M. tuberculosis* H37Rv was taken in a 1.5 ml micro centrifuge tube, washed twice with 500 μl of TE buffer-1 (50 mM Tris.Cl and 5 mM EDTA, pH 8.0) and suspended in 200 μl of TE buffer. The bacilli were heat killed at 80 °C for 1 h on a dry bath and were centrifuged at 7700 × g for 10 min. The resulting cell pellet was suspended in 200 μl of TE buffer, followed by addition of 26 μl of 10 mg/ml of lysozyme and 2 μl of 1 mg/ml of RNase to the suspension. This was incubated for 2 h at 37 °C. After incubation, 60 μl of 10% SDS and 1 μl of 20 mg/ml proteinase K were added to the cell suspension, mixed properly and further incubated at 60 °C for 1 h. 660 μl of 5 M NaCl and 550 μl of CTAB/NaCl solution (10% CTAB and 0.7 M NaCl) were added to the suspension and it was incubated for 30 min. After incubation, the cell debris were pelleted down by centrifugation at 9500 × g for 20 min at room temperature. The clear supernatant was taken in a fresh eppendorf tube and was extracted first with equal volume of phenol (50% v/v) /chloroform (49% v/v) /iso-amyl alcohol (1% v/v) and then with equal volume of chloroform (98% v/v) /iso-amyl alcohol (2% v/v). Finally, the DNA in the aqueous phase was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol and suspended in 100 μl of TE buffer-2 (10 mM Tris.Cl, 1 mM EDTA, pH 8.0). The concentration of DNA was estimated to be 50 ng/μl.

2.2.1.2. Isolation and amplification of the genes

The primers used for isolation and amplification of the genes by polymerase chain reaction (PCR) from the genomic DNA of *M. tuberculosis* H37Rv are given in the Table 1. The forward primers contain *NcoI* or *NdeI* and the reverse primer contained *HindIII* or *XhoI* restriction enzyme sites. Rv3874sRP and Rv3875sRP contain stop codon (5'- TCA - 3') immediately after the *HindIII* or *XhoI* sites, respectively. In a typical 50 μl PCR reaction, 10 ng of *M. tuberculosis* H37Rv genomic DNA was used; the concentration of
Table 1. The primers used for generation of wild type CFP-10 and ESAT-6

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Oligo Name</th>
<th>Oligo sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>Rv3874FP</td>
<td>5'-GGATccatggCAGAGATGAAGACCGATGC-3'</td>
</tr>
<tr>
<td>2</td>
<td>Rv3874RP</td>
<td>5'-CCTTaagcttGAAGCCCATTTGCGAGGAC-3'</td>
</tr>
<tr>
<td>3</td>
<td>Rv3874sRP</td>
<td>5'-CCTTaagcttTCAGAAGCCCATTTGCGAGGAC-3'</td>
</tr>
<tr>
<td>4</td>
<td>Rv3875FP</td>
<td>5'-GGAATTCCatagACAGAGCAGCAGTGGGAATTTCG-3'</td>
</tr>
<tr>
<td>5</td>
<td>Rv3875RP</td>
<td>5'-CTAGctcgagTGCGAACAATCCCGATCGAGCTTG-3'</td>
</tr>
<tr>
<td>6</td>
<td>Rv3875sRP</td>
<td>5'-CTAGctcgagTCATGCGAACACATCCCAGTGACGTTG-3'</td>
</tr>
</tbody>
</table>

forward and reverse primer were 5 \( \mu M \) (25 pmols) each and the concentration of each dNTP was 200 \( \mu M \). The reactions were carried out in 1x PCR buffer, 1x Q-buffer (Qiagen, Hilden, Germany) and distilled water. The PCR reactions were carried out using MJ Research PTC-150 MiniCycler (Global Medical Instrumentation, Inc.). Each reaction was given an initial denaturation step of 3 min at 95 °C which facilitated melting of the genomic DNA, followed by 25 cycles of denaturation at 94 °C for 1 min, primer annealing at 65 °C for 1 min, and primer extension at 72 °C for 1 min. Each PCR reaction was terminated with a final extension step of 72 °C for 20 min followed by cooling down to 4 °C. The size of the fragments was verified by running the PCR products on a 1.5% agarose gel in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer and comparing their sizes with 100 bp dsDNA NEB ladder. The PCR products were extracted from the gel by using Qiagen Gel extraction kit.

2.2.1.3. Restriction digestion and ligation of the genes in expression vectors

2 \( \mu g \) PCR product of Rv3874 was digested with 10 U of NcoI and 20 U of HindIII in 1x NEBuffer 2 for 2 h at 37 °C in a water bath. The reaction was continued for 10 h after addition of 5 U of NcoI and 10 U of HindIII. The digested products were run on 1.5% agarose gel and purified using Gel extraction kit. 2 \( \mu g \) pET28b vector was digested with 10 U of NcoI and 20 U of HindIII in 1x NEBuffer 2 for 2 h at 37 °C in a water bath. The digested products were run on 1.5% agarose gel and purified using the Gel extraction kit. 200 ng of NcoI–HindIII digested Rv3874 and pET28b were used to set a 15 \( \mu l \) ligation
reaction in 1× T4 DNA ligase buffer and 200 U of T4 DNA ligase from NEB on ice. The reaction was mixed properly and incubated at 16 °C for 12-16 h. The ligation mixture was then directly transformed into chemically competent (prepared using CaCl₂ method) *E.coli* DH5α cells. In a similar way the PCR product of Rv3874s was digested with *NcoI*-HindIII and ligated with *NcoI*-HindIII digested pQE60.

The PCR products Rv3875 and Rv3875s were initially cloned into pGEMT (Promega) by following user’s manual. The clones containing the genes in right orientation were selected by digesting pGEMT clones with *NdeI* and *NdeI*-XhoI. The correct clones were subsequently digested with *NdeI*-XhoI and run on 1.5% agarose gel to separate *NdeI*-XhoI digested Rv3875 or Rv3875s and were purified using the Gel extraction kit. The purified digested products were ligated with *NdeI*-XhoI digested pET22b vector.

2. 2.1.4. Confirmation of the clones

The positive clones were identified by restriction digestion of the clones with suitable restriction enzymes and analyzing the digested products on 1.5% agarose gel. Clones with required insert and vector sizes were selected as positive clones. These clones were subsequently verified by sequencing using T7 promoter primer at the DNA Sequencing Facility at UDSC, Department of Biochemistry, University of Delhi South Campus, New Delhi-110021.

2.2.2. Preparation of ESAT-6 and CFP-10 mutants

Mutants of ESAT-6 were prepared by using mismatched oligos as described by Higuchi et al. (1988). Briefly, a mutant primer (MP) and its complementary pair (CMP) containing the required change in base were chemically synthesized. Separate PCR reactions were performed using forward primer (FP) of ESAT-6 with CMP (PCR-I) and reverse primer (RP) of ESAT-6 with MP (PCR-II). The PCR products thus obtained were run on 2.0% agarose gel and purified using the Gel extraction kit. Another PCR reaction (PCR-III) was performed by mixing 0.1 ng of the purified products of PCR-I and PCR-II with ESAT-6 FP and RP primers, and the resulting PCR product contained the desired mutation. The PCR product was run on a 1.5% agarose gel for both verification of size and for purification. All PCR reactions were carried out in presence of Q-buffer (Qiagen). The
strategy of mutagenesis is described in the Figure I and the oligonucleotide primers used for construction of ESAT-6 mutants are tabulated in Table 2. The mutated PCR products were subcloned into pET22b as described above. Mutants for CFP-10 were prepared and cloned into pET28b using a similar method. The primers used for the construction of CFP-10 mutants are tabulated in Table 3. All mutant clones were verified by DNA sequencing using both T7 promoter primer and T7 terminator primer at the DNA Sequencing Facility at UDSC, New Delhi.

2.2.3. Over-expression and Purification of CFP-10 and ESAT-6 proteins

2.2.3.1. Over-expression and Purification of CFP-10 and its mutants

The vectors containing CFP-10 and CFP-10 mutants were transformed into BL21 (DE3) E. coli cells and grown in LB medium supplemented with kanamycin (50 \(\mu\)g/ml). The pQE60 vector containing untagged CFP-10 (pQE60-CFP-10SC) was transformed into E. coli M15 cells and was grown in LB medium containing both ampicillin (100 \(\mu\)g/ml) and kanamycin.

BL21 (DE3) cells containing the plasmid pET28b-CFP-10 were grown in LB medium to standardize optimum temperature, induction OD\(_{600}\), IPTG concentration and induction time for highest expression of soluble C-terminal hexa-histidine tagged CFP-10. During the standardization procedure, CFP-10 was expressed in a temperature range from 18 °C to 37 °C, OD\(_{600}\) was varied from 0.6 to 1.2, IPTG was varied from 0.1 mM to 2 mM, and the induction time was varied from 1 h to 12 h. The highest level of soluble CFP-10 was obtained by inducing the protein expression at OD\(_{600}\) = 1.0 with a final IPTG concentration of 600 \(\mu\)M and induction time of 3 h. The culture was immediately harvested and the pellet was suspended in the 50 mM NaH\(_2\)PO\(_4\) buffer containing 300 mM NaCl, 10 mM Imidazole, 2% Glycerol, 1mM PMSF, pH 7.5 (buffer A) at 4 °C. 20 ml of buffer A was used to suspend the pellet obtained from 1 l of culture. 20 \(\mu\)l of protease inhibitor cocktail (Sigma) was added to the cell suspension obtained from 1 l of culture. The cells were lysed by using Constant Cell Disruption System (Labmate (Asia) Pvt. Ltd.) at 26 kpsi at room temperature. Rest of the steps were performed at 4 °C. The lysate was centrifuged at 30,600 \(\times\) g for 1 h and the supernatant was passed through 0.2 \(\mu\)m syringe filters. The clear supernatant thus obtained was applied to a 7 ml Ni-NTA column pre-equilibrated
FIGURE I. Schematic representation of the experimental approach for the introduction of point mutation into the genes by PCR. Mutation was introduced in the PCR products of ESAT-6 and CFP-10 by using a pair of mutant primer and complementary mutant primer by the method described above.
Table 2. The primers used for generation of ESAT-6 mutants

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Oligo Name</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L29D-MP</td>
<td>5'- CGTCCATTCATCCCTTCGATGACGAGGGAAGCAGTC-3'</td>
</tr>
<tr>
<td>2</td>
<td>L29D-CMP</td>
<td>5'- GACTGCTTTCCCTCGTATCGAGGGAATGGAGCAG-3'</td>
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<tr>
<td>3</td>
<td>G32D-MP</td>
<td>5'- CATTCCCTCCTGAGGGAATGAATTTGAC-3'</td>
</tr>
<tr>
<td>4</td>
<td>G32D-CMP</td>
<td>5'- GGTCAGGGACTGCTTTATCTCCTCGATCAGGAGGGAATG-3'</td>
</tr>
<tr>
<td>5</td>
<td>A41D-MP</td>
<td>5'- GACCAAGCTCGCATGCTGGGCGGTAG-3'</td>
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<td>6</td>
<td>A41D-CMP</td>
<td>5'- CTACGCCAGGCATCTGCGAGCTTGTC-3'</td>
</tr>
<tr>
<td>7</td>
<td>L65D-MP</td>
<td>5'- GACGCCACCGGTACCGAGGATAAACACGCGCTGAG-3'</td>
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<td>8</td>
<td>L65D-CMP</td>
<td>5'- CTGCAGCGGTGTATTCTCGGTAGGCGTGTC-3'</td>
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</table>

Table 3. The primers used for generation of CFP-10 mutants

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Oligo Name</th>
<th>Oligo sequence</th>
</tr>
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<tr>
<td>1</td>
<td>I21R-MP</td>
<td>5'- GTAATTTCGAGCGCGCTCCGGCCGACCT-3'</td>
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<tr>
<td>2</td>
<td>I21R-MP</td>
<td>5'- CAGGTCGCGGAGCGCCGCTCGAATTAC-3'</td>
</tr>
<tr>
<td>3</td>
<td>L25R-MP</td>
<td>5'- CTCAGGCGACCAGGAAACCAGATCG-3'</td>
</tr>
<tr>
<td>4</td>
<td>L25R-MP</td>
<td>5'- CGATCCTGGGTTTCGCCGTGCCTGAG-3'</td>
</tr>
<tr>
<td>5</td>
<td>W43R-MP</td>
<td>5'- GCAGGGCCAGCGCGGTGGCGG-3'</td>
</tr>
<tr>
<td>6</td>
<td>W43R-MP</td>
<td>5'- CCGCAACCGCGCCCCTGCCCTGC-3'</td>
</tr>
<tr>
<td>7</td>
<td>F58R-MP</td>
<td>5'- GGTGGTGCCGACGGAAGCAG-3'</td>
</tr>
<tr>
<td>8</td>
<td>F58R-MP</td>
<td>5'- GCTGCTTCTTGGCAGCACCACC-3'</td>
</tr>
</tbody>
</table>
Materials and Methods

with buffer A. Unbound proteins were removed by washing the column with 20 column volumes (CVs) of buffer A containing 20 mM Imidazole. The bound CFP-10 was finally eluted with 4 CVs of buffer A containing 250 mM Imidazole. The column fractions were checked for purity on a 15% SDS-PAGE and the fractions containing more than 80% pure protein were pooled together. The pooled fractions were dialyzed against Q-sepharose buffer (20 mM NaH₂PO₄, 1 mM EDTA, 1 mM PMSF, pH 6.5) and loaded on a 10 ml Q-sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK) column pre-equilibrated with the same buffer. The protein was eluted in the flow through and was found to be more than 95% pure after analyzing on 15% SDS-PAGE. The purified protein was dialyzed against 20 mM NaH₂PO₄, 50 mM NaCl, 0.1% NaN₃, pH 6.5, and concentrated by ultrafiltration using the Amicon YM-3 (MWCO 3 kDa) ultrafiltration membrane. The concentrated protein was estimated using both the extinction coefficient method and Bradford method as described in the section 2.2.3.3, and was stored at 4 °C. Yield of the purified CFP-10 was 8 mg per litre of culture. The CFP-10 mutants were processed in a similar way and were dialyzed against 20 mM NaH₂PO₄, 50 mM NaCl, 1mM PMSF, pH 6.5 (buffer B) before storing at 4 °C.

*E. coli* M15 cells containing the plasmid pQE60-CFP-10SC were grown in LB medium to obtain untagged CFP-10 protein. Untagged CFP-10 was expressed in a similar way as described for hexa-histidine tagged CFP-10. After harvesting, the cell pellet was suspended directly in the buffer B containing protease inhibitor cocktail and lysed using cell disruptor. The lysate was centrifuged at 16060 × g for 30 min and the clear supernatant thus obtained was passed through 0.2 μm filter and was stored at 4 °C before use.

2.2.3.2. Over-expression and Purification of ESAT-6 and its mutants

The vectors containing ESAT-6 and ESAT-6 mutants were transformed into BL21 (DE3) *E. coli* cells and grown in LB medium supplemented with ampicillin (100 μg/ml). The pET22b vector containing untagged ESAT-6 was transformed into *E. coli* BL21(DE3) cells and was grown in LB medium containing ampicillin.

BL21 (DE3) cells containing the plasmid pET22b-ESAT-6 were grown in LB medium and optimum temperature, induction OD₆₀₀, IPTG concentration, and induction time conditions required to achieve highest expression of soluble C-terminal hexa-histidine
tagged ESAT-6 were standardized. However, it was not possible to obtain significant quantity of soluble ESAT-6 and, therefore, culture conditions were standardized for maximum level of expression of ESAT-6 in the insoluble inclusion bodies. The highest level of expression of ESAT-6 was obtained when the culture was induced at \( \text{OD}_{600} = 0.6 \) with a final concentration of 500 \( \mu \text{M} \) IPTG and induction period of 6 h. The cells were harvested at room temperature and the cell pellet from 1 l culture was suspended in 20 ml of 50 mM Tris.HCl, 1 mM PMSF, 1 mM EDTA, pH 8.0, and was stored in ice. 20 \mu l of protease inhibitor cocktail was added to the cell suspension. The cells were lysed by using Constant Cell Disruption System (Labmate (Asia) Pvt. Ltd.) at 26 kpsi at room temperature. Rest of the steps were performed at 4 °C. The lysate was centrifuged at 30,600 \( x \) g for 20 min. The pellet thus obtained contained inclusion bodies and cell debris. The inclusion bodies were dissolved in 50 mM NaH\(_2\)PO\(_4\), 8 M urea, pH 8.0 buffer (buffer C) and centrifuged again at 30,600 \( x \) g for 1 h. The supernatant obtained was passed through 0.2 \mu m syringe filter and was loaded on a 7 ml Ni-NTA column pre-equilibrated with buffer C. Unbound protein was removed by washing the column with 10 CVs of buffer C, pH 6.3 and 2 CVs of buffer C, pH 5.9. The bound ESAT-6 was eluted with 4 CVs of buffer C, pH 4.5. Immediately after elution, pH of the buffer containing the eluted protein was adjusted to neutral pH (pH=7.0). Fractions of >95% purity were pooled together and the protein was refolded by dialysis. In a typical refolding procedure, \( \approx 30 \) ml of eluted protein was dialysed against 2 l of dialysis buffer (25 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, 1 mM EDTA, and pH 6.5) at 4 °C. The dialysis buffer was first changed after 24 h, then after 36 h and the dialysis was stopped after 48 hours. The refolded ESAT-6 was subsequently dialyzed against NMR buffer (20 mM NaH\(_2\)PO\(_4\), 50 mM NaCl, 0.1% NaN\(_3\), pH 6.5), and concentrated by ultrafiltration through Amicon YM3 (MWCO 3 kDa) membrane. The concentrated protein was estimated using both the extinction coefficient method and Bradford method as described in the section 2.2.3.3, and was stored at 4 °C. Yield of purified ESAT-6 was \( \approx 30 \) mg per litre of culture. The sample was stored at 4 °C and was stable for several months. The ESAT-6 mutants were processed in a similar way and were dialyzed against 20 mM NaH\(_2\)PO\(_4\), 50 mM NaCl, 1mM PMSF, pH 6.5 before storing at 4 °C.
Soluble untagged ESAT-6 was expressed by growing the cells at 18 °C and inducing with 0.25 mM IPTG for 6 h (Kulshrestha et al., 2005). After harvesting, the cell pellet was suspended directly in the buffer B (20 mM NaH₂PO₄, 50 mM NaCl, 1mM PMSF, pH 6.5) containing protease inhibitor cocktail and lysed using cell disruptor. The lysate was centrifuged at 16060 × g for 30 min and the clear supernatant thus obtained was passed through 0.2 μm filter and was stored at 4 °C before use.

2.2.3.3. Determination of protein concentration

The concentration of CFP-10 and ESAT-6 proteins in aqueous protein samples were determined by extinsiton coefficient method and the Bradford colorimetric method.

For calculating concentration (C) of the proteins by extintion coefficient method, the formula given by Mach et al. (1992) was used.

\[
C = \frac{A_{280} - 10^{(2.5\log A_{320} - 1.5\log A_{350})}}{5540n_{trp} + 1480n_{tyr} + 134n_{s-s}}
\]

Where,
- \(A_{280}, A_{320}\) and \(A_{350}\) are optical density reading with a 1 cm path length at the indicated wavelength
- \(n_{trp}, n_{tyr}\) and \(n_{s-s}\) are the number of Trp, Tyr and Cys residues present in the protein molecule

The equation utilizes the molar extintion coefficients of the chromatophores tryptophan, tyrosine and cystine as 5540 M⁻¹cm⁻¹, 1480 M⁻¹cm⁻¹ and 134 M⁻¹cm⁻¹, repectively, at 280 nm. Since CFP-10 and ESAT-6 do not contain Cys residues, the molar absorbance of Cys i.e. 134\(n_{s-s}\) can be removed from the equation for calculating concentration of the proteins. This equation also includes appropriate correction contribution of non-protein spectral components and light scattering i.e. optical density values at \(A_{320}\) and \(A_{350}\). While estimating proteins, the aqueous protein sample was taken in a 1 cm path length quartz cuvette, and optical densities were recorded at 280 nm, 320 nm and 350 nm. These values were subtracted from the optical densities of only buffer at 280, 320 and 350 nm, respectively, and fitted to the formula to determine the accurate protein concentration. In a typical estimation procedure, the protein samples were diluted
to three different protein concentrations, estimated by the above described method and averaged to obtain the accurate protein concentration.

Protein concentrations were also determined by using Bradford reagent from Sigma (New Delhi, India) and following the user’s manual for the ‘Micro 2 ml assay protocol’.

The concentration of the proteins determined by both of the methods were found to be similar.

2.2.4. Isotopic labeling of CFP-10 and ESAT-6

Uniformly $^{15}$N or $^{13}$C, $^{15}$N-isotopically labeled ESAT-6 and CFP-10 were prepared by growing the cells in M9 minimal media containing either $^{15}$N-ammonium sulfate only or both $^{15}$N-ammonium sulfate and $^{13}$C-glucose, as the sole sources of nitrogen and carbon, respectively. Briefly, M9-agar plates supplemented with 30 µg/ml of kanamycin or 50 µg/ml of ampicillin were plated with *E. coli* BL21(DE3) transformed with pET28b-CFP-10 or pET22b-ESAT-6, respectively, and incubated at 37 °C. Visible colonies appeared after 15 h.

For preparation of $^{15}$N-labeled CFP-10, a single colony from the transformed M9 plate was inoculated in to 10 ml minimal media broth containing 1 g/l of $^{15}$N-ammonium sulfate as the sole nitrogen source, 10 g/l unlabeled dextrose and 30 µg/ml of kanamycin, and expression of the protein was checked. Upon confirmation of expression, the cells were grown in 2 l $^{15}$N-ammonium sulfate containing M9 media using the standardized procedure employed for expression of hexa-histidine tagged CFP-10 in LB medium. The cells were harvested, lysed, and the $^{15}$N-labeled CFP-10 was purified, concentrated and stored in the same way as described for unlabeled CFP-10. The same procedure was followed to prepare $^{13}$C, $^{15}$N-isotopically labeled CFP-10 except that the sole nitrogen and carbon sources were $^{15}$N-ammonium sulfate (1 g/l) and $^{13}$C- D-glucose (2 g/l).

For preparation of $^{15}$N-labeled ESAT-6, a single colony from the transformed M9 plate was inoculated to 10 ml minimal media broth containing 1 g/l of $^{15}$N-ammonium sulfate as the sole nitrogen source, 10 g/l unlabeled dextrose and 50 µg/ml of ampicillin, and expression of the protein was checked. Upon confirmation of expression, the cells were grown in 1 l $^{15}$N-ammonium sulfate containing M9 media using the standardized procedure employed for expression of hexa-histidine tagged ESAT-6 in LB medium. The
cells were harvested, lysed, and the $^{15}$N-labeled ESAT-6 was purified, concentrated and stored in the same way as described for unlabeled ESAT-6. The same procedure was followed to prepare $^{13}$C, $^{15}$N-isotopically labeled ESAT-6 except that the sole nitrogen and carbon sources were $^{15}$N-ammonium sulfate (1 g/l) and $^{13}$C-D-glucose (2 g/l). The uniformly $^2$H, $^{13}$C, $^{15}$N-isotopically labeled ESAT-6 was prepared by growing the cells in M9 medium containing D$_2$O in place of water, $^{15}$N-ammonium sulfate (1 g/l) and C-d$_7$, $^{13}$C D-glucose (2 g/l). Moreover, unlike the $^{15}$N or $^{15}$N and $^{13}$C labeling, where the M9 medium was sterilized by autoclaving, the $^2$H, $^{13}$C, $^{15}$N-labeled M9 medium was filter-sterilized and 0.1% CDN100-Bioexpress medium (Cambridge Isotope Ltd, Andover, MA, USA) was added to the culture medium before growing the cells. The cells were harvested, lysed, and the $^2$H, $^{13}$C, $^{15}$N-labeled ESAT-6 was purified, concentrated and stored in the same way as described for unlabeled ESAT-6.

Before concentrating, all labeled samples were dialyzed against the NMR buffer (20 mM NaH$_2$PO$_4$, 50 mM NaCl, 0.1% NaN$_3$, pH 6.5) passed through the chelating resins Chelex 100 sodium form (chelexed NMR buffer) to remove cations.

2.2.5. Isothermal Titration Calorimetry

Isothermal titration calorimetric (ITC) experiments were performed at 25 °C on a VP-ITC calorimeter from MicroCal™ (Northampton, MA, USA). The calorimeter was calibrated according to the user manual of the instrument. Stock solutions of ESAT-6 and CFP-10 were dialyzed extensively against the phosphate buffer (20 mM NaH$_2$PO$_4$, 50 mM NaCl, 1 mM EDTA, pH 6.5) and degassed for 20 min prior to each of the ITC experiments. Titrations were performed at least in duplicate using the same set of stock solutions. The ITC experiments were done by adding aliquots of CFP-10 to ESAT-6. The sample cell was filled with 1.3 ml of 0.042 mM of ESAT-6 (titrand) and titrated against CFP-10, which was filled in the syringe at a concentration of 0.42 mM. 65 injections with an injection volume of 4 µl each were made at an interval of 4 sec. During the titration, the reaction mixture was continuously stirred at 400 rpm. Control experiments were performed by injecting CFP-10 into buffer under conditions exactly similar to the ESAT-6/CFP-10 titration, to take into account ‘heats of dilution’ and ‘viscous mixing’. The heats of injections of control experiment were subtracted from the raw data of ESAT-6 and CFP-10
titration. The ITC data were analyzed using the ORIGIN version 7.0 software provided by Microcal. The heats of binding were normalized with respect to the titrant concentration and a volume correction was performed to take into account dilution of titrand during each injection. The amount of heat produced per injection was calculated by integration of the area under each peak using a baseline selected by the ORIGIN program.

2.2.6. Differential Scanning Calorimetry

Differential scanning calorimetric experiments were carried out on a VP-DSC calorimeter from MicroCal (Northampton, MA, USA). The calorimeter was calibrated according to the user’s manual of the instrument. The volume of reference and sample cells was 0.51 ml. Phosphate buffer (20 mM NaH₂PO₄, 50 mM NaCl, 1 mM EDTA, pH 6.5) was used in all experiments and a buffer scan was recorded before each sample run under exactly similar conditions. CFP-10—ESAT-6 complex was extensively dialyzed against phosphate buffer and brought to a concentration of 0.105 mM. Both the sample and buffer solutions were thoroughly degassed for 20 min just prior to the experiment. DSC scans were performed from 20 to 80 °C at four different heating rates of 20, 40, 60 and 90 °C per hour. After heating up to 80 °C, the samples were cooled to 20 °C and rescanned. Degassing during the experiment was prevented by additional constant pressure of 2 atm over the liquid solutions in the cells. Buffer scans were subtracted from the sample scans and the data was normalized with respect to protein concentration, scan-rate and electrical calibration of the calorimeter, to generate the excess heat capacity versus temperature thermogram of the sample. The pre- and post-transition baselines were selected for the thermogram with the Origin 7.0 program and the transition enthalpy, T_m, and T_{1/2} were determined by integration and non-linear curve fitting to a two-state model.

2.2.7. Circular Dichroism Spectroscopy

2.2.7.1. Recording CD spectra

CD measurements were carried out on a Jasco spectropolarimeter Model J-810 (Jasco International Co., Ltd, Tokyo, Japan) fitted with a thermostatically controlled cell holder having an accuracy of ± 0.1 °C. Calibration of the spectropolarimeter was performed with
(+)-10-camphorsulfonic acid. The CD results were expressed as mean residue ellipticity (MRE), in degree cm$^2$/dmol, which is defined as:

$$\text{MRE} = \frac{\theta \times 100 \times M_r}{c \times d \times N_A}$$

Where,

- $\theta$ = observed ellipticity in degree,
- $c$ = protein concentration in mg/ml,
- $d$ = path length in cm,
- $M_r$ = protein molecular weight,
- $N_A$ = number of amino acid residues.

The percentage of $\alpha$-helical content was determined by K2d program (www.embl-heidelberg.de/~andrade/k2d.html).

Isothermal wavelength scan of protein samples was carried out at indicated temperatures, with a scan rate of 10 nm/min in the wavelength range of 250-200 nm, with a response time of 1s, data pitch of 0.5, at protein concentrations within the range of 3 $\mu$M to 6 $\mu$M using a quartz cell of 2 mm path length. Three scans were averaged for each spectrum. All spectra were corrected by subtracting the buffer background. Phosphate buffer (20 mM NaH$_2$PO$_4$, 50 mM NaCl, 1 mM EDTA, pH 6.5) was used during the recording of CD spectra.

2.2.7.2. Study of thermal unfolding/refolding using CD

Thermal denaturation studies were performed by recording spectra of protein samples at various temperatures ranging from 25 °C to 70 °C, with a 5 °C increment. Samples were incubated for 10 min at each temperature before recording the spectra.

Change in mean residue ellipticity values at 222 nm represents change in helical content in proteins. Since two-thirds of CFP-10—ESAT-6 complex is in helical conformation, fractional helicities observed at MRE values at 222 nm corresponds to fraction of protein folded during the thermal heating or cooling process. Therefore, the fraction of protein folded was calculated by the equation:
Where,

\[ \frac{[\theta]^{\text{obs}} - [\theta]^{\text{den}}}{[\theta]^{\text{nat}} - [\theta]^{\text{den}}} \]

\[ [\theta]^{\text{obs}} \] is the experimentally observed mean residue ellipticity at 222 nm, 
\[ [\theta]^{\text{nat}} \] and \[ [\theta]^{\text{den}} \] are mean residue ellipticities at 222 nm when the protein is in its native state (at 25 °C, in phosphate buffer) and in its fully denatured state (at 70 °C, in phosphate buffer) (Muhle-Goll et al., 1995).

CD unfolding curves were produced by plotting fraction of protein folded vs. temperature. Each thermal denaturation experiment was repeated at least twice with fresh samples. In all cases, after the heating experiment, the samples were tested for their transparency.

2.2.7.3. Recording CD spectra in presence of lipid vesicles and detergent micelles

To study the effect of lipid vesicles on the conformation of proteins, far-UV CD spectra of the protein samples were recorded in the presence of 0.5 mM dimyristoyl-DL-\( \alpha \)-phosphatidylcholine (DMPC) vesicles (Sigma, USA). 1 mg of DMPC was dissolved in chloroform and was dried under a continuous stream of nitrogen to form a film on the inner walls of a glass test tube. The lipid film was vacuum dried for 2 hours and suspended in phosphate buffer. The suspension was incubated at 37 °C for 30 min, and was vortexed and sonicated with a 3 mm probe (Bransonic, Danbury, CT, USA) at room temperature till a clear solution was observed. The sample was centrifuged for 10 min at 16060 x g at room temperature to remove Titanium particles. The supernatant was taken out and was used for the CD experiments.

A stock solution of 200 mM dodecyl phosphocholine (DPC) was prepared by dissolving dried DPC in phosphate buffer (20 mM NaH\(_2\)PO\(_4\), 50 mM NaCl, 1 mM EDTA, pH 6.5). It was centrifuged at 16060 x g at room temperature to remove any suspended particles. Isothermal wavelength scans of protein samples containing 20 mM DPC were carried out as described in the section 2.2.7.1.
2.2.8. NMR Spectroscopy

2.2.8.1. HSQC spectra of free and complex proteins

NMR spectra were recorded with 1 mM protein samples taken in chelexed NMR buffer containing 5% v/v H2O, on a Varian 600 MHz spectrometer equipped with triple nuclei inverse probe, at 30 °C. 2D [15N-1H] HSQC spectra were recorded for following samples:

1) 15N-labelled CFP-10,
2) 15N-labelled ESAT-6,
3) 15N-labelled CFP-10 — unlabelled ESAT-6,
4) unlabelled CFP-10—15N-labelled ESAT-6,
5) 15N-labelled CFP-10 — 15N-labelled ESAT-6.

The HSQC spectrum for each experiment was acquired with 1024 and 128 complex points in the 1H and 15N dimensions, respectively. The 2D data was zero filled to 2048 and 1024 points along 1H and 15N dimensions, respectively, and apodized with 60° shifted sine-square-bell window function along both dimensions prior to Fourier transformation. The spectral data were processed using FELIX 2002 (Accelrys, Bangalore, India) and analyzed using XEASY (Bartels et al., 1995)

2.2.8.2. Thermal scanning of CFP-10—ESAT-6 complex

For thermal unfolding or refolding studies, 2D [15N-1H] HSQC spectra with 1 mM 15N-labeled CFP-10—15N-labeled ESAT-6 complex were recorded on Bruker 500 MHz spectrometer at 30, 40, 50, 55, 60 and 65 °C followed by HSQC spectra on the same sample were recorded at 60, 55, 50, 40 and 30 °C. The HSQC spectrum for each experiment was acquired with 1024 and 128 complex points in the 1H and 15N dimensions, zero filled to 2048 and 1024 points, respectively, and apodized with 60° shifted sine-square-bell window function along both dimensions prior to Fourier transformation. The spectral data were processed using FELIX 2002 and analyzed using XEASY.
2.2.8.3. NMR assignments for ESAT-6
Backbone assignments for ESAT-6 were obtained for a sample of 1:1 complex of $^2$H, $^{13}$C, $^{15}$N-labelled ESAT-6 and $^{13}$C, $^{15}$N-labelled CFP-10 at 30 °C, from triple resonance experiments HNCA, HNCO, HN(CA)CB and HN(CA)CO, and $^{15}$N-edited NOESY-HSQC. The time domain data was acquired with 512, 64, and 32 points in the $^1$H, $^{13}$C and $^{15}$N dimensions, respectively. The fids were zero filled to 2048, 512 and 128 points. All dimensions were apodized with a 60° shifted squared sine-bell window function and Fourier transformed. The spectral data were processed using FELIX 2002 and analyzed using XEASY.

2.2.8.4. $^{15}$N-edited NOESY-HSQC
$^{15}$N-edited NOESY-HSQC experiment was performed with a mixing time of 150 ms. The time domain data was acquired with 1024, 80, and 32 points in the $^1$H (direct), $^1$H (indirect), and $^{15}$N dimensions, respectively. The fids were zero filled to 1024, 512 and 128 points. All dimensions were apodized with a 60° shifted squared sine-bell window function and Fourier transformed. The spectral data were processed using FELIX 2002 and analyzed using XEASY.

2.2.9. Fluorescence Spectroscopy
Fluorescence spectra were acquired at 25 °C, on a Perkin Elmer Life Sciences LS 5B spectroluminescence meter (USA) or Varian Cary Eclipse fluorescence spectrophotometer (USA) fitted to a water bath, using a 5 mm path length quartz cell. 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) stock solution was prepared in phosphate buffer and the concentration of ANS was determined using extinction co-efficient of $\varepsilon = \varepsilon = 8000 \text{ M}^{-1}\text{cm}^{-1}$ at 372 nm (Muro-Pastor et al., 2003). The samples were kept in dark immediately after addition of ANS stock solution to the proteins, and measurements were made within an hour. The ANS binding experiments were carried out with the excitation maximum of ANS (380 nm) and emission spectra were recorded in the range of 400 to 600 nm, with a slit width of 12 nm for excitation and 10 nm for emission. The concentration of the protein samples was 10 μM and the molar ratio of protein and ANS was 1:10 in all experiments.
2.2.10. Limited Proteolysis and Western Blotting

For the limited proteolysis experiment, in a 500 µl reaction mixture, 40 µM of ESAT-6, CFP-10, and 1:1 complex of CFP-10—ESAT-6 were separately incubated with 1 µl of 1 mg/ml of trypsin at 4 ºC. The reactions were performed in 20 mM Na$_2$HPO$_4$, 50 mM NaCl, pH 7.6. 50 µl aliquots of each reaction were taken out at time points of 0, 1, 5, 20, 40, 60, 120, and 180 min. The proteolysis reactions were stopped by precipitating proteins with 10% TCA and then the samples were analyzed on a 15% SDS-PAGE.

The fragments obtained on the SDS-PAGE were transferred to a nitrocellulose membrane by applying 350 mA current for 4 hours in Tris-glycine-methanol buffer (7.26g Tris, 33.78g glycine, 600 ml methanol, 2400 ml distilled water). During the transfer process, buffer was continuously stirred. The transferred bands on the membrane were initially detected by Ponceau S staining. After destaining the membrane, it was blocked with skimmed milk (prepared in 1x Phosphate buffered saline or PBS) and rabbit anti-His antibody was added to it. Composition of 10 × PBS was 0.137 M NaCl, 0.027 M KCl, 0.032 M Na$_2$HPO$_4$, 0.015 M K$_2$HPO$_4$ and 0.015 M KH$_2$PO$_4$, volume of the buffer was made upto 1 l with distilled water. Excess of antibody was removed by washing the membrane with 1x PBS containing 0.05% Tween 20. The membrane was subsequently treated with HRP conjugated secondary antibody. Excess of secondary antibody was removed by washing with 1x PBS Tween 20 buffer. The blot was developed by 10 ml of 1 × PBS containing 10 mg 3, 3'-diaminobenzidine tetrahydrochloride, 10 mg imidazole and 6 µl H$_2$O$_2$.

2.2.11. CFP-10 and ESAT-6 pull down assays

The CFP-10 and ESAT-6 pull down assays were performed in a 0.5 ml Ni-NTA column. The column was equilibrated with buffer B (20 mM NaH$_2$PO$_4$, 50 mM NaCl, 1mM PMSF, pH 6.5). For the CFP-10 pull down assay, 0.5 mg of refolded tagged ESAT-6 dialyzed in buffer C was passed through the column, followed by washing with the same buffer. Excess of *E. coli* lysate containing untagged CFP-10 was allowed to pass through the column. To remove unbound proteins, the column was washed extensively with 20 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM Imidazole, 1mM PMSF, pH 6.5 (buffer C). The bound proteins were eluted with buffer C containing 250mM imidazole. In a similar way, binding
of untagged CFP-10 with the mutants of ESAT-6 was tested individually on the Ni-NTA column. The eluted fractions were analyzed on a 15% SDS-PAGE.

The ESAT-6 pull down assay was performed in a similar way. 0.5 mg of C-terminally hexa-histidine tagged CFP-10 was passed through the equilibrated column, followed by washing with the same buffer. Excess of *E. coli* lysate containing untagged ESAT-6 was allowed to pass through the column. To remove unbound proteins, the column was washed extensively with the buffer C and the bound proteins were eluted with buffer C containing 250 mM imidazole. The same procedure was followed to examine the binding of untagged ESAT-6 with the mutants of CFP-10. The eluted fractions were analyzed on a 15% SDS-PAGE.

2.2.12. Lymphocyte Proliferation assay

Venous blood was collected in a heparinized tube (10U per ml of blood) from a healthy BCG vaccinated human and peripheral blood mononuclear cells (PBMCs) were separated using Ficoll density gradient by centrifugation. All steps were performed in sterilized conditions. Briefly, 5 ml of blood was slowly overlaid on a 15 ml falcon tube containing 3 ml Ficoll and centrifuged at 2500 × g for 1 hr at 25 ºC. After centrifugation RBCs, PBMCs, and the plasma were separated out as lower, interphase and upper layer, respectively. The interphase layer (∼3ml) was collected very carefully and diluted with equal volume of RPMI in a 15 ml falcon tube and mixed by inverting the tube for 5 or 6 times. The sample was centrifuged at 500 × g for 5 min at room temperature. The supernatant was discarded completely and the pellet was suspended in 12 ml of RPMI by tapping and pipetting slowly. This was centrifuged at 400 × g for 5 min at room temperature. The pellet thus obtained was suspended in 1 ml of RPMI 1640 containing 10% fetal bovine serum and cell density was counted under a microscope using a hemocytometer.

3.5 × 10⁵ cells in 220 µl were seeded in 96-well flat-bottom tissue culture plates. Triplicate cultures were stimulated with 5 µg ESAT-6 or CFP-10 or mutant proteins and 10 µg of CFP-10—ESAT-6 complex or mutant complexes. Three culture-wells were stimulated with 1 µg of phytohemmaglutinin which served as positive control for lymphocyte proliferation and three culture-wells were left untreated. After addition of the
antigens, the culture plate was incubated at 37 °C in a humidified chamber containing 5% CO₂ for 4 days. After 72 h of incubation, 0.5 μCi [³H]thymidine was added to each well. After 96 h, supernatants were collected from each culture wells and stored at −80 °C. The cells were harvested on glass fiber filter paper (Skatron, Sterling, USA) and the incorporated radioactivity was measured in a liquid scintillation counter (Beckman Coulter LS 6500, Beckman Coulter, Inc., Fullerton, CA, USA) and were expressed in counts per minute (c.p.m.). The results were presented in stimulation index (S.I.) which was calculated as:

\[
S.I. = \frac{\text{c.p.m. of antigen stimulated culture}}{\text{c.p.m. of unstimulated culture}}
\]

2.2.13. Cytokine assays.
The culture supernatants stored in −80 °C were thawed on ice, centrifuged to remove any particulate materials and used for cytokine assays. The cytokines IFN-γ and TNF-α were quantified by commercially available ELISA kit following recommended assay procedures provided by the manufacturer in VERSAmax microtiterplate reader (Molecular Devices, Sunnyvale, CA, USA). The sensitivity limits for IFN-γ was 4.7 pg/ml and TNF-α was 7.8 pg/ml.