Chapter 3:

RESULTS
3.1. EXPRESSION AND PURIFICATION OF CFP-10 AND ESAT-6 PROTEINS

CFP-10 was expressed in soluble form and was purified using Ni-NTA chromatography followed by Q-sepharose chromatography. The purified CFP-10 protein was collected as the flow through of the Q-sepharose column. CFP-10 was found to be more than 95% pure and the yield of the protein was 6 to 8 mg per litre of culture. ESAT-6 was expressed as inclusion bodies which were solubilized using 8M urea and purified up to 95% in a single step using Ni-NTA chromatography. After purification urea was removed and the protein was refolded by dialysis. The yield of purified ESAT-6 was \( \approx 30 \) mg per litre of culture. The cloning strategy added a sequence of thirteen residues (Lys-Leu-Ala-Ala-Ala-Leu-Glu-His-His-His-His-His-His) at the C-terminal of CFP-10 and a sequence of eight additional residues (Leu-Glu-His-His-His-His-His-His) at the C-terminal of ESAT-6. CFP-10 consists of 113 residues (\( M_r = 12,315 \) Da) and ESAT-6 consists of 103 residues (\( M_r = 10,970 \) Da).

3.2. BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION OF CFP-10 AND ESAT-6

3.2.1. Thermodynamic parameters governing CFP-10 and ESAT-6 complex formation

Isothermal titration calorimetry (ITC) experiments were carried out to accurately measure the association constant for ESAT-6 and CFP-10 complex formation. The raw ITC data, generated by titration of 1.3 ml of 0.042 mM ESAT-6 with 50 injections of 4 \( \mu l \) of 0.42 mM CFP-10 is shown in Fig. 1A. The integrated areas under each peak versus molar ratio of ESAT-6 to CFP-10 are plotted in Fig. 1B. The binding isotherm of ESAT-6 with CFP-10 is characterized by strong heat release, which is indicated by a slope approaching infinity. The heat released decreases as ESAT-6 gets saturated. In the last 23 injections of the titration, only heat of dilution is observed. The binding isotherm in Fig. 1B was fitted to a single-site binding model for determination of thermodynamic parameters. The solid line indicates best fit to the plot. The parameters used in fitting were the stoichiometry of association (n), the binding constant (\( K_B \)), and the change in enthalpy (\( \Delta H_B \)). The values of these parameters obtained from the non-linear least-square fit to the
FIGURE 1. Typical calorimetric isothermal titration measurements of the interaction of CFP-10 with ESAT-6 in phosphate buffer at 25 °C. (A) shows raw data of heat effect (in μcal/sec) of sixty five 4 μl injections of 0.42 mM CFP-10 into 1.3 ml of 0.042 mM ESAT-6 performed at 4 s interval. (B) the data points (●) were obtained by integration of heat signals plotted against the molar ratio of ESAT-6 to CFP-10 in the reaction cell. The solid line represents a calculated curve using the best-fit parameters obtained by a non-linear least squares fit. The heat of dilution was subtracted from the raw data of titration of CFP-10 with ESAT-6.
Results

The ITC binding isotherm can be characterized by a unitless value c (Pierce et al., 1999), which is given by 
\[ c = K_B [M] n; \]
where \( K_B \) is binding constant; [M], concentration of the macromolecule [ESAT-6], and \( n \) the stoichiometry of the reaction. For an accurate determination of binding constant, a ‘c’ value between 1 and 1000 is recommended. In case of ESAT-6 and CFP-10, the value of ‘c’ is 840, which is indicative of a tightly bound complex. The free energy change (\( \Delta G \)) associated with complex formation is given by: 
\[ \Delta G = -RT \ln K_B; \]
where \( R \) is the gas constant and \( T \) is the temperature in Kelvin. At 25 °C, \( \Delta G \) for complex formation is -9.95 kcal/mol. The entropy change associated with complex formation is determined from the equation: 
\[ \Delta G = \Delta H - T\Delta S. \]
At 25 °C, \( \Delta S \) is -101 cal/mol K. Both the entropy change and enthalpy change associated with complex formation are characteristically high. However, typical enthalpy-entropy compensation results in a moderate value of \( \Delta G \) of -9.95 kcal/mole. The free energy change for complex formation between ESAT-6 and CFP-10 is comparable to the \( \Delta G \) associated with similarly sized protein-protein interactions e.g. \( \Delta G \) of -9.6±0.5 kcal/mol was observed for interaction between turkey ovomucoid third domain with \( \alpha \)-chymotrypsin and \( \Delta G \) of -11.3±0.7 kcal/mol was observed for interaction between T-cell factor 4 and \( \beta \)-catenin (Filfil et al., 2003 and Knapp et al., 2001).

3.2.2. Thermal unfolding of CFP-10—ESAT-6 complex is completely reversible

Differential scanning calorimetry (DSC) studies were carried out to assess the thermal stability of the CFP-10—ESAT-6 complex and to accurately measure the enthalpy and heat capacity changes involved in the unfolding. DSC thermogram of the thermal unfolding of the complex at a concentration of 0.105 mM in phosphate buffer and a scan rate of 60 °C / hour, from 20 to 80 °C, is shown by the solid line curve in Fig. 2. After the first heating scan, the sample was cooled from 80 to 20 °C and then a second heating scan was recorded, which is shown by the dotted line curve in Fig. 2. The peak shaped thermograms indicate cooperativity during unfolding (Jelesarov et al., 1999). The thermal unfolding transition is characterized by an enthalpy change (\( \Delta H \)) of 69 kcal/mol, \( T_m \) of 53.4 °C, and \( T_{1/2} \) of 9.01 °C. However, no change in heat capacity (\( \Delta C_p \)) was observed for
FIGURE 2. Thermal reversibility of 1:1 CFP-10—ESAT-6 complex monitored by DSC. DSC thermogram of 0.51 ml of 0.105 mM 1:1 CFP-10—ESAT-6 complex from 20 °C to 80 °C, at a scan rate of 60 °C/hour. The raw data were baseline corrected for buffer. The plots show excess heat capacity as a function of temperature in °C. The complex was heated to 80 °C for the first thermogram shown by (—). The sample was then cooled down to 20 °C. The second thermogram recorded by re-heating the same sample again is shown by (-----).
the thermal unfolding transition. DSC scans recorded at scan rates of 20, 40, 60 and 90 °C/hour showed only a small shift in the Tm from 54 to 53.4 °C and a small decrease in transition enthalpy from 74 to 69 kcal/mol. As the first and second heating scans are completely overlapping at every scan rate, it strikingly indicates that the thermal unfolding of the complex is completely reversible.

The secondary and tertiary structural changes associated with thermal unfolding of the complex were followed by steady state Circular Dichroism (CD) and 2D [\textsuperscript{15}N-\textsuperscript{1}H] HSQC Nuclear Magnetic Resonance (NMR) experiments, respectively. Far-UV CD spectra of CFP-10, ESAT-6, and CFP-10—ESAT-6 complex were similar to those reported previously by Renshaw et al. (2002). Since CFP-10 is almost completely unstructured, the thermal unfolding and refolding experiments were performed only for ESAT-6 and the complex. Steady-state CD scans were recorded on a sample first at increasing temperatures in the range of 25 to 75 °C, and then in decreasing order from 75 to 25 °C, at 5 °C intervals. The thermal unfolding and refolding profiles of ESAT-6 and the complex are shown in Fig. 3A. The midpoints of thermal unfolding transitions (Tm) of ESAT-6 and the complex are at 33 °C and 53 °C, respectively. For the complex, the Tm determined from CD (53 °C) matches well with that determined by DSC (53.4 °C). CD spectra recorded before and after unfolding, at 25 °C, for ESAT-6 and the complex are shown in Fig. 3B and 3C, respectively. Similar to the unfolding and refolding profiles mentioned above, entire CD spectra before and after unfolding overlapped at every temperature, suggesting that the molecular steps leading to thermal unfolding are retraced upon refolding for both ESAT-6 and the complex.

2D [\textsuperscript{15}N-\textsuperscript{1}H] HSQC spectrum serves as a fingerprint of the overall structure of a protein. The HSQC spectrum recorded with \textsuperscript{15}N-labelled CFP-10 at 30 °C is shown in Fig. 4A. The spectrum is characterized by sharp but narrowly dispersed peaks along the \textsuperscript{1}H\textsuperscript{N} dimension (within 7 – 8.5 ppm), which is consistent with CFP-10 being unstructured in its native form. The 2D [\textsuperscript{15}N-\textsuperscript{1}H] HSQC spectrum of \textsuperscript{15}N-labelled ESAT-6 is shown in the Fig. 4B. Broad peaks and peak dispersion pattern in the HSQC spectrum are consistent with the previously reported molten globular state of free ESAT-6. The HSQC spectrum of the complex formed between \textsuperscript{15}N-labelled CFP-10 and unlabelled ESAT-6 is shown in Fig. 4D, while that of the complex formed between \textsuperscript{15}N-labelled ESAT-6 with unlabelled CFP-10 is
FIGURE 3. Thermal reversibility of unfolding of ESAT-6 and 1:1 CFP-10–ESAT-6 complex monitored by CD. (A) Normalized transition curves for temperature-induced transition of ESAT-6 and the complex monitored at far-UV CD region at 222 nm. Thermal unfolding (○) and thermal refolding (△) profile of ESAT-6 and thermal unfolding (Δ) and thermal refolding (◇) profile of the complex were plotted as fraction of protein folded versus temperature in °C. (B) far-UV CD spectrum of ESAT-6 (○) was recorded in phosphate buffer, pH 6.5 at 25 °C. The sample was heated to 70 °C and cooled down to 25 °C and far-UV CD spectrum was recorded again (○). (C) CD spectrum of the 1:1 complex at 25 °C was recorded before thermal unfolding (○) and after thermal refolding (◇) as described for ESAT-6.
FIGURE 4. Conformational change observed individually in ESAT-6 and CFP-10 on complex formation. (A) and (D) show \[^{15}\text{N}-^{1}\text{H}\] HSQC spectra of \(^{15}\text{N}\)-labeled CFP-10 in free state and in complex with unlabelled ESAT-6, respectively. (B) and (E) show \[^{15}\text{N}-^{1}\text{H}\] HSQC spectra of \(^{15}\text{N}\)-labelled ESAT-6 in free state and in complex with unlabelled ESAT-6, respectively. (C), \[^{15}\text{N}-^{1}\text{H}\] HSQC spectrum of 1:1 \(^{13}\text{C}, {^{15}\text{N}}\)-labelled CFP-10—\(^{13}\text{C}, {^{15}\text{N}}\)-labelled ESAT-6 complex. (F), a spectrum produced by addition of the spectra (D) and (E). All spectra were recorded in NMR buffer (See Experimental Procedures) containing 5% (v/v) D\(_2\)O at 30 °C on a 600 MHz NMR spectrometer.
FIGURE 5. Thermal reversibility of 1:1 CFP-10—ESAT-6 complex monitored by NMR spectroscopy. 1 mM 1:1 $^{15}$N-labelled CFP-10—$^{15}$N-labelled ESAT-6 complex in NMR buffer, pH 6.5, with 5% (v/v) D$_2$O was used for monitoring thermal reversibility of the complex. [$^{15}$N-$^1$H] HSQC spectra were recorded on a 500 MHz NMR spectrometer at 30 °C (A), 40 °C (C), 50 °C (E), 55 °C (G), 60 °C (I) and 65 °C (K), in increasing order, after which [$^{15}$N-$^1$H] HSQC spectra on the same sample were recorded at 60 °C (J), 55 °C (H), 50 °C (F), 40 °C (D), and 30 °C (B), in decreasing order.
shown in the Fig. 4E. Fig. 4C shows 2D \(^{15}\text{N}-^{1}\text{H}\) HSQC spectrum of the complex in which both the proteins are \(^{13}\text{C},^{15}\text{N}\)-labelled. The sum of HSQC spectra of individually labelled proteins in complex i.e., the sum of spectra in the Fig. 4D and 4E, is shown in the Fig. 4F. Spectrum in the Fig. 4F overlaps very well with the spectrum of complex shown in Fig. 4C. In order to find any change in tertiary structure of the complex during the unfolding and refolding process, \(^{15}\text{N}-^{1}\text{H}\) HSQC spectra on 1 mM complex in phosphate buffer were first recorded at 30, 40, 50, 55, 60 and 65 °C, in increasing order (Fig. 5A, C, E, G, I and K, respectively), following which HSQC spectra on the same sample were recorded at 60, 55, 50, 40, and 30 °C (Fig. 5I, H, F, D and B, respectively), in decreasing order. The data shows that the tertiary structure is retained up till 60 °C. Strikingly, the peaks in HSQC spectrum at any particular temperature before and after unfolding are almost completely overlapping, and are representative of the HSQC spectrum of the complex, but not HSQC spectra of individual proteins ESAT-6 and CFP-10. This indicates that the tertiary structure of the complex is also completely regained after thermal unfolding.

3.2.3. Molecular recognition between ESAT-6 and CFP-10 exists even when the two proteins are in unstructured form

Since the secondary structure of ESAT-6 is highly dependent on the temperature, we investigated whether any residual secondary structure of ESAT-6 is necessary for complex formation with CFP-10. CD scans were recorded for samples in which ESAT-6 and CFP-10 were mixed at 25, 30, 35, 40, 45, 50 and 55 °C, and compared with CD scans of the complex formed between the two proteins at 25 °C and heated to equivalent temperatures. The Fig. 6 shows thermograms generated by plotting mean residue ellipticity at 222 nm as a function of temperature for ESAT-6, CFP-10, 1:1 complex of CFP-10—ESAT-6, and equimolar CFP-10 and ESAT-6 mixed at different temperatures. As can be seen from the figure, increase in helical content equivalent to that of the complex is observed when ESAT-6 and CFP-10 were mixed together at temperatures up to 55 °C, indicating formation of helices locally by interactions between specific segments of CFP-10 and ESAT-6. These results indicate that the secondary structure of ESAT-6 is not necessary for the complex formation and specific molecular recognition between the
FIGURE 6. Temperature dependence of the interaction of ESAT-6 and CFP-10. Isothermal CD spectra were recorded at 5 °C temperature intervals from 25 to 55 °C. The figure shows plot of mean residue ellipticity values at 222 nm as a function of temperature, recorded for ESAT-6 (○), CFP-10 (○), and 1:1 ESAT-6:CFP-10 complex formed by mixing equimolar proteins at 25 °C (△), and equimolar ESAT-6 and CFP-10 mixed together at 25, 30, 35, 40, 45, 50 and 55 °C (●).
interacting segments of ESAT-6 and CFP-10 exists even when the two proteins are in unstructured form.

3.2.4. CFP-10 reduces its susceptibility to trypsin digestion on forming complex with ESAT-6

To investigate the biochemical stability of the proteins, limited proteolysis with trypsin was performed at 4 °C for ESAT-6, CFP-10, and the 1:1 CFP-10—ESAT-6 complex, and the digested products thus obtained were analyzed on 15% SDS-PAGE. The Coomassie stained SDS-PAGE gels are shown in Fig. 7A. Upon trypsinolysis, CFP-10 showed multiple bands on SDS-PAGE after 1 min of digestion at 4 °C, and was completely digested to oligopeptides in 20 min. ESAT-6 was stable for 60 min at 4 °C. Further degradation of ESAT-6 yielded two bands corresponding to molecular weights of 14 kDa and 3 kDa. The 14 kDa band could be an aggregate of trypsin degraded products of ESAT-6. In contrast to ESAT-6 and CFP-10, the complex displayed a characteristic pattern upon trypsinolysis. On treatment of the complex with trypsin at 4 °C, one additional band appeared after 1 min incubation. The largest and smallest of these bands corresponded to CFP-10 and ESAT-6, respectively. A third band labeled as trCFP-10 (for truncated CFP-10), in between CFP-10 and ESAT-6, with molecular weight ~ 2 kDa lower than CFP-10 was observed, which apparently results from truncation of CFP-10 by cleavage at a particular site by trypsin. Upon continued incubation, the intensity of the band corresponding to CFP-10 decreased, while that of trCFP-10 increased with time and no change in the intensity of band corresponding to ESAT-6 was observed. After 2 h of trypsin treatment, the band corresponding to intact CFP-10 had disappeared completely, while the bands corresponding to trCFP-10 and ESAT-6 were still present. Essentially similar pattern of bands was observed for the complex after 3 h of trypsinolysis except that a weak band having an apparent mass of 6 kDa was observed, which resulted from further degradation of trCFP-10. Both ESAT-6 and CFP-10 have C-terminal hexa-histidine tags. The products of trypsinolysis experiments were probed by Western blotting with anti-His antibody. Western blot with anti-His antibody are shown in Fig. 7B. trCFP-10 is not detected on Western blotting with anti-His antibody, indicating that it results from cleavage of the C-terminal of CFP-10. Overall, these results indicate that complex
FIGURE 7. Limited proteolysis with trypsin of ESAT-6, CFP-10 and 1:1 CFP-10—ESAT-6 complex. (A) SDS-PAGE of aliquots taken out at different time points for reaction of 40 μM of ESAT-6, or CFP-10, or 1:1 CFP-10—ESAT-6 complex with 1 μg of trypsin at 4°C. The lanes 1, 4, 7, 10, 13, 16, 19 are for CFP-10; lanes 2, 5, 8, 11, 14, 17, 20 are for ESAT-6; and the lanes 3, 6, 9, 12, 15, 18, 21 are for the 1:1 CFP-10—ESAT-6 complex, and correspond to aliquots withdrawn after 0, 1, 5, 20, 60, 120 and 180 min of trypsinosylation reaction. LMW is low molecular weight protein marker. (B) Western blot developed with anti-His antibody. The lanes of the blot correspond to the lanes of SDS-PAGE, except for LMW.
formation leads to interdependent protection of an N-terminal fragment of CFP-10 and ESAT-6 from trypsinolysis.

3.2.5. ESAT-6 possesses solvent exposed hydrophobic clusters

In order to assess the solvent exposed hydrophobic surface of the proteins, we have studied the change in fluorescence intensity of 8-anilino-1-naphthalenesulfonate (ANS) upon binding to ESAT-6, CFP-10, and the 1:1 CFP-10—ESAT-6 complex. Fig. 8 shows extrinsic fluorescence spectra of ANS in the presence of ESAT-6, CFP-10, and the complex, at 25 °C. The fluorescence intensities have been normalized with respect to the maximum fluorescence intensity of ANS bound to ESAT-6. As expected from its molten globule state, ESAT-6 showed high ANS binding. No change in fluorescence intensity of ANS was observed in the presence of CFP-10, indicating that ANS did not bind to CFP-10, as expected from the unstructured form of CFP-10. A decrease of 65 ± 5% in ANS fluorescence intensity was obtained upon CFP-10—ESAT-6 complex formation.

3.2.6. DMPC vesicles stabilize the secondary structure of ESAT-6 above its melting temperature

To investigate the binding of ESAT-6, CFP-10, and the complex to lipid membranes, 6 µM of protein samples were incubated with dimyristoyl-DL-α-phosphatidylcholine (DMPC) vesicles in phosphate buffer and the change in conformation was monitored by CD spectroscopy. CD spectra of CFP-10, 1:1 CFP-10—ESAT-6 complex, and ESAT-6 in the absence and presence of DMPC vesicles are shown in Fig. 9. At 25 °C, the CD spectra of CFP-10 and the complex did not show any significant change, while ESAT-6 showed a minor increase in helicity i.e. from 49% to 52%, in the presence of DMPC vesicles. On increasing temperature of the sample to 37 °C, CFP-10 and the complex still showed no change. However, ESAT-6 retained α-helical content of 32% in contrast to 19% α-helical content observed for ESAT-6 in the absence of DMPC vesicles at 37 °C. On cooling the same ESAT-6/DMPC vesicles sample from 37 °C to 25 °C, the α-helical content increased further to 63%, which is significantly higher than the helicity that was obtained on mixing ESAT-6 and DMPC vesicles at 25 °C. We further studied the
FIGURE 8. Binding of ANS to CFP-10, ESAT-6, and 1:1 CFP-10—ESAT-6 complex.
The fluorescence emission spectra of 100 μM ANS in presence of 10 μM of ESAT-6 (○), CFP-10 (□) and 1:1 CFP-10—ESAT-6 complex (▲) in phosphate buffer, pH 6.5, at 25 °C.
FIGURE 9. Far-UV CD spectra of CFP-10, 1:1 CFP-10–ESAT-6 complex, and ESAT-6 in the presence of DMPC vesicles. CD spectra of 6 μM CFP-10, 1:1 CFP-10–ESAT-6 complex, and ESAT-6 without DMPC vesicles in phosphate buffer, pH 6.5, at 25 °C (○) and 37 °C (●) and with DMPC vesicles in phosphate buffer, pH 6.5, at 25 °C (△) and 37 °C (▲) are shown. The spectra obtained at 25 °C after cooling the protein samples containing DMPC vesicles from 37 °C, are shown with symbols (▽).
FIGURE 10. Study of effect of DPC on CFP-10, ESAT-6, and CFP-10—ESAT-6 complex using circular dichroism. Far-UV CD spectra of CFP-10 (○), ESAT-6 (△) and 1:1 mixture of ESAT-6 and CFP-10 (○) recorded in phosphate buffer, pH 6.5, with and without 20 mM dodecylphosphorylcholine (DPC) at 25 °C.
effect of 20 mM dodecylphophorylcholine on individual proteins and the complex at 25 °C (Fig. 10). The α-helical content of CFP-10, ESAT-6, and the complex, in the presence of DPC were 47%, 61% and 54%, respectively. When the samples of CFP-10 and ESAT-6 in 20 mM DPC were mixed together and CD spectrum of the resulting sample was recorded, we obtained a similar spectrum like the spectrum of CFP-10–ESAT-6 complex in 20 mM DPC.

3.2.7. Interaction of ESAT-6 mutants with CFP-10

We have used a novel approach to select residues for mutations from the 26 residues of ESAT-6 that are at the interface between ESAT-6 and CFP-10 in the complex, as reported by Renshaw et al. (2005). Our approach was based on detection of NOEs from the backbone amide protons of ESAT-6 to the side-chain protons of CFP-10. Residues of ESAT-6, the amide protons of which showed strongest NOEs with the side chain protons of CFP-10 in the labeled complex, were selected for mutation. For detecting NOEs, we prepared the complex from $^{13}$C, $^{15}$N-labelled CFP-10 and $^2$H, $^{13}$C, $^{15}$N-labelled ESAT-6. A set of 3D triple resonance experiments HNCO, HNCA, and HN(CA)CB were recorded to validate our sample. Strips from HNCA and HN(CA)CB spectra demonstrating the sequential assignments of residues Leu39 to Trp43 are shown in Fig. 11A and B, respectively. These assignments are similar to those reported by Renshaw et al. (2004). $^{15}$N-edited NOESY-HSQC spectrum was recorded for the complex for detecting the NOEs. NOEs from backbone amide protons of ESAT-6 and side chain protons of CFP-10 were observed for the segments Ala14-Ala15-Ser16 (1.187 ppm), Ala17-Ile18 (1.200 ppm), Ser24-Ile25 (0.934 ppm), Leu28-Leu29-Asp30 (0.897 ppm), Glu31-Gly32-Lys33-Gln34-Ser35-Leu36 (0.745 ppm), Leu39-Ala40-Ala41-Ala42-Trp43 (0.808 ppm), and Glu64-Leu65-Asn66 (1.415 ppm). Values in parenthesis are the chemical shift of the side chain protons of CFP-10 with which backbone amide proton of ESAT-6 show NOE. Fig. 11C shows NOE between the amide protons for the segment Leu39 to Trp43 from ESAT-6 to the side chain proton of CFP-10. Strongest NOEs were observed for the residues Leu29, Gly32, Ala41 and Leu65. Based on this, four point mutants L29D, G32D, A41D and L65D of ESAT-6 were generated. We have studied complex formation between ESAT-6 mutants
FIGURE 11. Sequential assignments and inter-protein NOEs for a segment of ESAT-6 interacting with CFP-10. (A) and (B) are strips showing the sequential assignments from 3D HNCA and HN(CA)CB spectra, respectively, recorded from 1 mM, 1:1 complex of $^2$H, $^{13}$C, $^{15}$N-labelled ESAT-6 and $^{13}$C, $^{15}$N-labelled CFP-10 in NMR buffer with 5% (v/v) D$_2$O at 30 °C on a 600 MHz NMR spectrometer. The strips are taken at the indicated $^{15}$N chemical shifts that were assigned to residues 39 to 43 of ESAT-6. They are centered about the corresponding amide proton chemical shifts. The top of the sequence specific assignments is indicated by one letter amino acid code and by sequence number. The one directional arrows in these figures indicate a sequential walk through 2D $^{13}$C$_\alpha$-$^1$HN and $^{13}$C$_\beta$-$^1$HN planes taken in position of corresponding $^1$HN, $^{15}$N, $^{13}$C$_\alpha$ and $^1$HN, $^{15}$N, $^{13}$C$_\beta$ resonances in 3D HNCA and HN(CA)CB spectra, respectively. (C) shows strips from $^1$H, $^{15}$N- NOESY-HSQC spectrum recorded with $\tau_{\text{mix}}$ of 150 ms. In these strips, NOEs are shown between downfield amide protons and upfield aliphatic protons. The amide protons correspond to the sequentially assigned segment 39 to 43 of ESAT-6. The backbone amide protons of this segment show NOEs with protons at 0.808 ppm from a side chain of CFP-10.
FIGURE 12. Study of complex formation between ESAT-6 mutants and CFP-10 using CFP-10 pull-down assay. A 15% SDS-PAGE showing results of CFP-10 pull-down assay. LMW is low molecular weight protein marker. Rest of the lanes show purified ESAT-6 or ESAT-6 mutants and Ni-NTA eluate (see Experimental Procedures).
FIGURE 13. Study of complex formation between ESAT-6 mutants and CFP-10 using circular dichroism. Far-UV CD spectra of CFP-10 (○), ESAT-6 mutants (△) and 1:1 mixture of ESAT-6 mutant and CFP-10 (○) recorded in phosphate buffer, pH 6.5, at 25 °C.
FIGURE 14. Thermal reversibility of 1:1 CFP-10—ESAT-6 complex and 1:1 CFP-10—ESAT-6-A41D complex monitored by CD. Normalized transition curves for temperature-induced transition of CFP-10—ESAT-6 and CFP-10—ESAT-6-A41D complexes monitored at far-UV CD region at 222 nm. Thermal unfolding (Δ) and thermal refolding (○) profile of CFP-10—ESAT-6 complex and thermal unfolding (□) and thermal refolding (○) profile of the CFP-10—ESAT-6-A41D complex were plotted as fraction of protein folded versus temperature in °C.
and CFP-10 by CFP-10 pull-down assays and CD spectroscopy. In parallel, we have also studied the interaction of ESAT-6 mutants with DMPC membranes by CD spectroscopy.

SDS-PAGE of CFP-10 pull down assay is shown in the Fig. 12. As can be seen from the figure, two prominent low molecular weight bands corresponding to untagged CFP-10 and tagged ESAT-6 (or ESAT-6 mutants) were observed in the eluted fractions of ESAT-6, ESAT-6-G32D, and ESAT-6-A41D. A single prominent band corresponding to the molecular weight of ESAT-6 was observed in the eluted fraction of ESAT-6-L29D and ESAT-6-L65D. This indicates that only the ESAT-6 mutants G32D and A41D form complex with CFP-10.

The CD spectra of ESAT-6 mutants in absence and in presence of equimolar CFP-10 are shown in Fig. 13. The α-helical contents of ESAT-6 mutants L29D, G32D, A41D and L65D were 13%, 18%, 46% and 9% as compared to 49% α-helicity of ESAT-6. In presence of equimolar CFP-10, the α-helical contents for the mixture containing ESAT-6 mutants L29D, G32D, A41D and L65D were 42%, 61%, 64% and 12% respectively as compared to 64% α-helical content of wild CFP-10—ESAT-6 complex. The Fig. 14 shows a comparative study of thermal unfolding and refolding of CFP-10—ESAT-6 and CFP-10—ESAT-6-A41D complexes.

3.2.8. Interaction of ESAT-6 mutants with phospholipid membranes.

The CD spectra of ESAT-6 mutants in presence and absence of DMPC vesicles at 37 °C are shown in the Fig. 15. The α-helical content of L29D, G32D, A41D, and L65D at 37 °C without DMPC vesicles were 15%, 10%, 19% and 9% respectively, and with DMPC vesicles were 18%, 14%, 30% and 15% respectively. This shows that, only A41D mutant of ESAT-6 shows behavior similar to the wild ESAT-6, while other mutations affect the interaction of ESAT-6 with DMPC membranes.

3.2.9. Interaction between ESAT-6 and CFP-10 mutants

Based on the interaction of ESAT-6 and its mutants L29D, G32D, A41D, and L65D with CFP-10 described above and the solution structure of CFP-10—ESAT-6 complex, we selected I21R, L25R, and F58R mutants of CFP-10, which are expected to strongly interact with the mutated residues of ESAT-6. In addition, W43R mutant of CFP-
FIGURE 15. Interaction of ESAT-6 mutants with DMPC vesicles. The figures show far-UV CD spectra of 6 μM ESAT-6 mutants recorded at 37 °C, without DMPC vesicles (○) and with 0.5 mM DMPC vesicles (□) in phosphate buffer, pH 6.5.
was constructed in order to examine the role of highly conserved Trp43 in the ESAT-6 family of proteins (Pallen MJ, 2002, and Brodin et al., 2005). The interactions among the wild-type and mutant ESAT-6 and CFP-10 proteins were studied using ESAT-6 pull-down assay and CD spectroscopy.

The results of the pull-down assay are shown in the Fig. 16. Two prominent bands corresponding to the molecular weight of hexa-His tagged CFP-10 and untagged ESAT-6 were observed in the eluates of wild type CFP-10, CFP-10-I21R, and CFP-10-W43R. Only a single prominent band corresponding to the molecular weight of hexa-His tagged CFP-10 was observed in the eluates of CFP-10-L25R and CFP-10-F58R. This indicates that I21R and W43R mutants of CFP-10 interact strongly with ESAT-6 while the mutants L25R and F58R do not form complex with ESAT-6.

The interactions among the wild-type and mutant ESAT-6 with the CFP-10 mutants were further studied using circular dichroism. All CD spectra are displayed in the Fig. 17. The α-helical content of the complexes were determined from K2d program. The percentages of α-helical content of all the wild-type–mutant and mutant–mutant complexes of ESAT-6 and CFP-10 are summarized in the Table 1. In general, among the 25 combinations of wild-type and mutant CFP-10 and ESAT-6 proteins, L25R and F58R point mutants of CFP-10 and L29D and L65D point mutants of ESAT-6 did not form complex. On the other hand, I21R and W43R mutants of CFP-10 and G32D and A41D mutants of ESAT-6 were involved in formation of 1:1 heterodimeric complex.

**TABLE 1.** Percentage of helical content obtained from CD spectra of 1:1 combinations of wild type or mutant CFP-10 and ESAT-6.

<table>
<thead>
<tr>
<th></th>
<th>CFP-10</th>
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<tbody>
<tr>
<td></td>
<td>wt</td>
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<tr>
<td><strong>ESAT-6</strong></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>61</td>
</tr>
<tr>
<td>L29D</td>
<td>42</td>
</tr>
<tr>
<td>G32D</td>
<td>61</td>
</tr>
<tr>
<td>A41D</td>
<td>64</td>
</tr>
<tr>
<td>L65D</td>
<td>12</td>
</tr>
</tbody>
</table>
FIGURE 16. Study of complex formation between CFP-10 mutants and ESAT-6 using ESAT-6 pull-down assay. ESAT-6 pull-down assay was performed by affinity binding of hexa-His tagged CFP-10 or CFP-10 mutants to the Ni-NTA column and allowing *E. coli* whole cell lysate containing untagged ESAT-6 to pass through the column. The column was subsequently washed to remove unbound proteins and the proteins bound to the column were eluted with 250 mM Imidazole. The eluates, along with the purified CFP-10/CFP-10 mutants, were analyzed on a 15% SDS-polyacrylamide gel and are shown in the above figure. LMW is low molecular weight protein marker. 2nd lane is *E. coli* whole cell lysate containing untagged ESAT-6, and rest of the lanes are purified CFP-10/CFP-10 mutants and Ni-NTA eluates.
Results 93

FIGURE 17. Study on complex formation between CFP-10 mutants with wt-ESAT-6 and ESAT-6 mutants. Far-UV CD spectra of 4 or 6 μM 1:1 mixture of CFP-10-I21R with ESAT-6 (●), ESAT-6-L29D (○), ESAT-6-G32D (▲), ESAT-6-A41D (□) and ESAT-6-L65D (○). The CD spectra were recorded in phosphate buffer, pH 6.5, at temperature 25 °C, and are shown in the upper left panel of the figure. In similar conditions, far-UV CD spectra were recorded for 1:1 mixtures of CFP-10-L25R, CFP-10-W43R, and CFP-10-F58R with ESAT-6 or ESAT-6 mutants, and are shown in the lower left, upper right and lower right panel of the figure, respectively.
3.3. IMMUNOLOGICAL CHARACTERIZATION OF CFP-10 AND ESAT-6 PROTEINS: COMPARISON OF Th1 RESPONSE OF WT-CFP-10 AND ESAT-6 WITH MUTANT CFP-10 AND ESAT-6

Induction of Th1, but not Th2 response, has been known to provide better protective efficacy against tuberculosis. A bulk of data reveals that ESAT-6 and CFP-10 are potential inducer of Th1 response in animal models. We have analyzed the Th1 response of wild type and mutant ESAT-6 and CFP-10 proteins. In addition, we examined the Th1 response of wild-type 1:1 complex, six complex-forming mutants i.e. CFP-10–ESAT-6-G32D, CFP-10–ESAT-6-A41D, CFP-10-I21R–ESAT-6, CFP-10-I21R–ESAT-6-A41D, CFP-10-W43R–ESAT-6, and CFP-10-W43R–ESAT-6-A41D, and two non-complex-forming mixtures of mutants i.e. CFP-10-I21R–ESAT-6-L29D and CFP-10-F58R–ESAT-6-L65D. PHA was used as a positive indicator of T-cell proliferation.

The antigen induced lymphocyte proliferation was assessed by \(^{3}\text{H}\)thymidine incorporation into the DNA of the proliferating cells. The overall lymphocyte proliferation responses are presented in the Figure 18A. The proliferative activity of different antigens were compared using S.I. PHA, the potential T-cell inducer, showed the highest proliferation (S.I. = 24.2±0.7) among all of the antigens tested. The proliferative response shown by the wt-CFP-10 was almost half of the response of wt-ESAT-6 (S.I.s were 3.1±0.1 and 5.2±0.5, respectively), whereas the wt-CFP-10–ESAT-6 complex showed response similar to CFP-10. Upon analyzing individual mutant proteins, we found the S.I. of CFP-10-F58R was close to the S.I. of wt-CFP-10, while the CFP-10-I21R, L25R, and W43R mutants showed higher responses than wt-CFP-10. For the ESAT-6 mutants, the S.I. of G32D and L65D were close to that of ESAT-6, whereas it was lower for ESAT-6-A41D (S.I. = 2.9±0.8) and almost negligible for ESAT-6-L29D (S.I. = 1.4±0.1). Among the six complexes forming combinations, CFP-10-I21R–ESAT-6 showed a better response with S.I. of 5.2±1.2, while the remaining complexes showed response in the range of 2.5 to 3.5, as shown in the Figure 18A. Among the non-complex-forming combinations, CFP-10-F58R–ESAT-6-L65D showed better response (S.I. = 4.7±0.1) than the CFP-10-I21R–ESAT-6-L29D (S.I. = 2.4±0.1).
The Th1 interleukins IFN-γ and TNF-α are the key mediators for killing of phagocytized mycobacteria. Therefore, we determined the quantity of these interleukins secreted to the culture medium during the proliferation assay. The interleukin profiles are shown in the Figure 18B. All antigens were found to be potential inducers of IFN-γ. The wt-CFP-10 induced 284±8 pg/ml; wt-ESAT-6 induced 384±8 pg/ml; and the wt-CFP-10–ESAT-6 complex induced 391±11 pg/ml of IFN-γ from the PBMCs. The CFP-10 mutants I21R, L25R, and W43R could induce more IFN-γ secretion (nearly 373-381 pg/ml) than the wt-CFP-10 and CFP-10-L58R (216±7 pg/ml). Among the ESAT-6 mutants, ESAT-6-L29D was a poor inducer of IFN-γ (247±10 pg/ml), whereas rest of the ESAT-6 mutants induced IFN-γ secretion in the range of 383-396 pg/ml. All six complex forming combinations induced almost similar quantity of IFN-γ (in a range of 399-412 pg/ml). For the non-complex-forming mutant mixtures CFP-10-I21R–ESAT-6-L29D and CFP-10-F58R–ESAT-6-L65D, the IFN-γ secretion was 377±12 and 392±12 pg/ml, respectively.

CFP-10 produced almost negligible TNF-α, i.e. 4 ± 0.6 pg/ml, whereas wt-ESAT-6 and wt-CFP-10–ESAT-6 were strong inducer of TNF-α (472±27 and 591±42 pg/ml, respectively). Compared to the wt-CFP-10, the CFP-10 mutants I21R, L25R, and W43R produced high quantity of TNF-α (223±33, 185±29, and 228±50 pg/ml, respectively). However, the CFP-10-F58R was also poor inducer of TNF-α (68±31 pg/ml). All the four ESAT-6 mutants induced less TNF-α than the wt-ESAT-6. The lowest TNF-α secretion was shown by ESAT-6-G32D (26±14 pg/ml). Among the six mutant complexes CFP-10-I21R–ESAT-6 and CFP-10-W43R–ESAT-6 induced high level of TNF-α i.e. 559±16 and 534±52 pg/ml, respectively, while the remaining mutant complexes showed TNF-α secretion in the range of 142-365 pg/ml. The non-complex-forming mutant mixtures CFP-10-I21R–ESAT-6-L29D and CFP-10-F58R–ESAT-6-L65D secreted 245±30 and 77±24 pg/ml of TNF-α, respectively.
FIGURE 18. Comparison of the immune response to wild type and mutant ESAT-6, CFP-10 and CFP-10–ESAT-6 complex. PBMCs were isolated from a BCG vaccinated donor and stimulated for 96 h with individual wild type and mutant ESAT-6 and CFP-10, and with wild type-mutant and mutant-mutant complexes of ESAT-6 and CFP-10. Each bar in the panels represents an average ± SD of triplicate wells. (A) Lymphocyte proliferation and (B) IFN-γ and TNF-α detected in culture supernatant after the antigenic stimulation. For clarity, ESAT-6 and CFP-10 are abbreviated as ‘E’ and ‘C’ respectively, in this figure.