

CHAPTER IV

DISCUSSION

Many recombinant proteins have been successfully produced using *E. coli* which is the best-characterized host with many available expression systems. However, *E. coli* can not produce some proteins possessing complex tertiary and quaternary structure, the multiple disulfide bonds or other post-translational modifications to reach a native, biological active conformation. Producing these proteins in *E. coli* can be challenged. The delivery of protein across the cytoplasmic membrane into the periplasm of *E. coli* has been applied in the production of a number of heterologous proteins. The oxidizing environment in the periplasm promotes the correct formation of disulfide bonds (Makrides, 1996). Moreover, the periplasmic space appears to have less protease activity than in cytoplasm. The vast majority of proteins is exported by the common Sec pathway and therefore is responsible for the targeting and assembling of most proteins in phage display. Phage display is based on genetic fusion of proteins or peptides to gp III or gpVIII of filamentous bacteriophage. These fusion proteins are exported *via* the Sec machinery in an unfolded state into the periplasm, where they become incorporated into the phage particle. However, not every recombinant protein can be successfully inserted into the phage particle because of the limitation of the general phage display which depends on the Sec translocation pathway. Chimeric proteins that included large peptides or stretches of hydrophobic residues acting as stop-transfer sequences that

might be prevented from crossing the membrane result in the inability to be displayed by phage on their surface. Moreover, these obstructed proteins can be jammed the export channel which causes the formation of inclusion bodies of precursor that accumulates in an export-competent form. In addition, proteins that require a cytoplasmic environment and/or cytoplasmic components for folding are not suitable for the Sec pathway. To alleviate this problem, various methods have been applied such as modulating the culturing condition (Chappel *et al.*, 1998; Soltes *et al.*, 2003) and co-expression of chaperones involved in translocation and protein folding (Bothmann and Pluckthun, 1998; DeLisa *et al.*, 2004). However, other factors i.e. folding kinetics of individual molecules had not been solved. The expressed proteins, which completely fold in the cytoplasm, would not be facilitated by the Sec channel.

In 1998, an alternative secretory system, the twin-arginine translocation, was reported (Weiner *et al.*, 1998). The success in translocation of active green fluorescence protein (GFP) via the Tat pathway, which previously failed with Sec machinery, has been described (Thomas *et al.*, 2001). GFP is known to be stably folded, therefore it can be transported across the plasma membrane because Sec pathway is incapable of exporting folded proteins of this size. Hence the Tat pathway may be useful in phage displaying system, particularly for those proteins that may be folded before they can reach the Sec machinery or that contain complex disulfide bonds.

In this study, the external domain of CD147 molecule was selected to be a candidate of TorA twin-arginine leader fusion protein. The novel phagemid, pTat8-CD147, containing the TorA signal sequence was constructed to employ the Tat machinery for targeting and exporting to facilitate displaying the CD147Ex. In pTat8-CD147 construction, the parental phagemid (pComb8-CD147Ex) was modified by

replacing the signal sequence from PelB with that from TorA. The site-directed mutagenesis of pComb8-CD147Ex was performed to create the specific restriction site upstream PelB signal sequence because it has no restriction site in this region. The MutPelBa and MutPelBb mutagenic primers were used to make the *Nsi* I restriction site upstream PelB. Since the emergence of PelB at two places in pComb8-CD147Ex, thus double *Nsi* I restriction sites were generated in MpComb8-CD147Ex after the site-directed mutagenesis processes. The site-directed mutagenesis does not make newly synthesized strand covalently closed. Consequently, two staggered nicks appear at the synthetic ends of MpComb8-CD147 and it divide MpComb8-CD147 into two fragments, 844 bp containing PelB signal sequence, CD147Ex and gpVIII gene, and 3016 bp bearing ampicillin resistant gene. After transforming MpComb8-CD147 into *E. coli*, the nicked phagemids were repaired by bacterial host but not every phagemid can be successfully repaired. Most of resistant colonies are 3,016 bp-containing clones because the clones which carry the fragment of 844 bp can not be survived on LB agar containing ampicillin. The 2,942 bp of MpComp8-CD147Ex derived by digesting MpComp8-CD147Ex of 3016 bp with *Nsi* I and *Xba* I was used to ligate with Tat-CD147 fragment. The generated phagemid, pTat8-CD147, was transformed and the fusion gene was inducible expressed in *E. coli* TG-1. The CD147Ex was successfully expressed *via* the major coat protein, gpVIII, on phage particles. The occurrence of CD147 epitopes was evaluated by five mAbs in sandwich ELISA with HRP-conjugated anti-gpVIII mAb as a tracer (Figure 3.8). This system detected CD147 epitopes presented on phage particles and free form CD147Ex-gpVIII. As hypothesized, CD147Ex-gpVIII fusion proteins secreted through Tat machinery showed higher positive reactivity with all anti-CD147 mAbs when

compared with those delivered *via* the Sec pathway. The folding of two distinct epitopes of CD147 delivered *via* the Tat pathway was more efficient as demonstrated by M6-2B1 and M6-2F9. Interestingly, a strong positive signal was also obtained from TG-1 Δ *tatABC* mutant transformed with pTat8-CD147. Even though *tat* was deleted the TorA-CD147Ex-gpVIII fusion protein could pass through the membrane by non-specific pathway.

We further investigated whether the positive signal was due to the secretory or phage anchoring form of CD147Ex-gpVIII. To address this question, the expression of CD147Ex on the phage surface was evaluated by sandwich ELISA using the biotinylated anti gpIII mAb/HRP-conjugated anti-biotin antibody detection system. The absorbance values obtained from Φ Tat-CD147gpVIII and Φ Sec-CD147gpVIII were considerably distinct in most of mAbs tested. Since the positive signal determined the phage bound form of CD147Ex, the number of CD147Ex molecules presented in Φ Tat-CD147gpVIII was assumed to be higher than in Φ Sec-CD147gpVIII. Since gpVIII size is small, we presumed that the structure of gpVIII might be distorted upon folding along with its fusion partner, CD147Ex. Consequently, the transformed gpVIII lost the efficiency to anchor in the inner membrane, thus, unable to assemble into the phage particle. For Φ Tatmut-CD147gpVIII, there was no binding of phage to the solid phase coating with mAbs. In contrast, Φ Tatmut-CD147gpVIII showed the comparable binding activity with Φ Tat-CD147gpVIII when detected with HRP-conjugated anti-gpVIII mAb detection system. We speculated that after the TG-1 Δ *tatABC* mutant was infected with VCSM13 helper phage the recombinant phage progeny could not be generated. This finding corresponded to the former studies describing the defect in the integrity of *E.*

E. coli tat mutant outer membrane (Bruser and Sanders, 2003; Ize *et al.*, 2003). The *tat* mutants showed leakage of periplasmic protein, while the wild type *E. coli* did not (Stanley *et al.*, 2001). Regarding the periplasmic leakage and the defect of outer membrane, CD147Ex-gpVIII fusion proteins that accumulated in the cytoplasm as well as cytoplasmic membrane were directly released to the culture supernatant. Since they did not pass through a specific targeting/translocation system, the CD147Ex-gpVIII was not inserted in the inner membrane. Hence the majority of CD147Ex-gpVIII detected in the culture medium from the Δ *tat* strain was in a phage non-packaging form. Therefore, TorA-CD147Ex-gpVIII could be translocated to the periplasm and integrated to the membrane only *via* complete Tat machinery. This phenomenon could be confirmed by normal phage biology, as the progeny phage will gather only the gpVIII, which is translocated by specific pathway and correctly inserted into the membrane.

Very recently, Paschke *et al.* applied the Tat pathway for delivering a protein of interest to link to the phage particle (Paschke and Hohne, 2005). The fusion partner of displayed polypeptide was not gpVIII but Fos instead. Consequently, the exported protein distributed in the periplasmic space in a soluble form. In contrast, our findings demonstrate the incorporation of Tat-delivered heterologous protein into phage with the fusion partner gpVIII, which contained a hydrophobic region for incorporating into the *E. coli* inner membrane. Our observations here further support the idea that the Tat translocon is involved in membrane protein insertion (Hatzixanthis *et al.*, 2003).

The CD147Ex-gpVIII fusion protein in phage solutions was also identified by Western immunoblotting. The correct size of the CD147 fusion protein at 28 kDa was

observed from Φ Tat-CD147gpVIII which was identical to Φ Sec-CD147gpVIII (Intasai *et al.*, 2003). The immature polypeptide band did not appear on the membrane. This result confirmed the suitable delivery of expressed TorA-CD147-gpVIII and the specific processing of the TorA signal peptide. The reactive band at 28 kDa also appeared in lane loaded with Φ Tatmut-CD147gpVIII. Regarding this finding and sandwich ELISA results (Figure 3.8 and Figure 3.9), the CD147gpVIII contained in phage preparation from TG-1 Δ tatABC mutant culture was in free form. Although nonspecifically accessing the periplasm, TorA signal peptide was apparently removed from the recombinant molecule.

Various functional proteins such as tissue plasminogen activator derivative, antibody fragment (Fab and scFv) and human growth hormone were successfully displayed on phage particles (Bass *et al.*, 1990; Hoogenboom *et al.*, 1991; Manosroi *et al.*, 2001; McCafferty *et al.*, 1990; Sidhu *et al.*, 2000; Weiss and Sidhu, 2000). Proteins or peptides are fused to either gpVIII or gpIII. Generally, gpIII is the protein of choice for most phage display due to its tolerance for large insertion. However, its disadvantage is that less than one copy of recombinant protein is typically displayed per phage particle and is not suitable when functional avidity is required. Therefore, gpVIII phage displaying multivalent molecule was more preferable. In our preliminary study, the Φ Sec-CD147gpVIII demonstrated the binding to U937 cells in contrast to Φ Sec-CD147gpIII. However, gpVIII phage display is limited to the length of insert because the large insert can be less efficiently packaged into phage. The large insert might affect the initiation of phage assembly by disturbing the interaction between gpVIII and gpVII (Endemann and Model, 1995) or it might be too large in diameter to pass through the gpIV exit pore in the outer membrane (Marciano *et al.*,

1999). Recently, Phage Shock protein (PspA) was described in enhancing the efficiency of protein transportation *via* the Tat machinery (DeLisa *et al.*, 2004). The PspA is overexpressed in membrane-associated stress conditions including filamentous phage infection. The precise function of PspA is not clearly understood, but it appears to maintain the proton motive force (Kleerebezem *et al.*, 1996), which is solely required in Tat translocon function. Moreover, PspA may be affecting any of a number of protein translocation processes, including, the folding of the protein into a form competent for export, proteolysis of membrane associated translocation-incompetent precursors, or even release of the newly translocated protein from the periplasmic side of the membrane. Overall, the Tat pathway appears more suitable than the Sec pathway in phage display applications.

Here we solved the limitation in using gpVIII as a fusion partner. The multivalent phage displaying the precise structure of CD147Ex is being applied in further studies for uncovering its signal transduction and ligand-partner roles. The current finding also identified an unconventional route of filamentous phage packaging system that is independent of Sec components. Moreover, the structural domains of CD147Ex molecule are homologous to immunoglobulin; expression by pTat8 could improve the quality of the antibody libraries. This strategy overcomes the aggregation that results in non-secreting of ScFv or Fab, and thus, not attached to phage particle. Consequently, the possibility to obtain the reactive clones in the panning step will be increased.