CHAPTER 4

DISCUSSION

Since phage display technology was invented in 1985 (Smith, 1985), certain investigations have demonstrated that phage display is a high potential technology for producing functional recombinant proteins. This technique was used as an effective way for producing large numbers of diverse peptides and proteins and molecules that perform specific functions (Barbas, 1993; Griffith, 1993; Winter *et al.*, 1994; Burton, 1995; Ladner, 1995; Neri *et al.*, 1995; Hoogenboom, 1998; Ladner, 2000; Seigel, 2001, Tayapiwatana and Kasinrerk, 2002). This technique can also be used to study protein-ligand interactions (Cesareni, 1992), receptor and antibody-binding site (Griffith, 1993; Winter *et al.*, 1994; Appenzeller *et al.*, 2001; An *et al.*, 2002), and to improve or modify the affinity of proteins for their binding partners (Burton, 1995; Ladner, 1995; Neri *et al.*, 1995). Phage display technology is used to display the molecule of allergens on phage particle as libraries for screening the patient. Serum IgE from allergic patient is immobilized and specific binding to the allergen on phage particle from libraries. This method is found to reduces the time required for the selection of candidate clones to a few weeks (Appenzeller *et al.*, 2001). Moreover, phage display is applied to use for displaying the antibody fragment (scFv) against GPIIIa integrin. The V_H and V_L genes of mAb against GPIIIa integrin are cloned into the phagemid expressing vector and linked together by linker sequence. The recombinant vector is transformed into *E. coli* and subsequently cultured for

phage display. The specificity of scFv phage is assayed by ELISA. For its application, ADP-induced platelet aggregation can be inhibited by ScFv fragment in a dose-dependent manner and the maximal inhibition rate was obtained at a concentration of 750 nM. In addition, the ScFv fragment has ability to inhibit the binding of fibrinogen to platelets and react with endothelial cells. (An *et al.*, 2002).

Recently, Tayapiwatana and Kasinrerk have applied this technique to generate phage expressing a leukocyte surface molecule, CD99 (Tayapiwatana and Kasinrerk, 2002). The CD99 cDNA from the mammalian expressing vector was amplified by PCR and subcloned into the phagemid expression vector, pComb3HSS. The recombinant phagemid vector, pCom3H-CD99, was transformed into *E. coli* strain XL-1 Blue and subsequently cultured for phage display. The sandwich ELISA was performed for detection the CD99 which display on phage particle. In the inhibition experiment, the CD99-Φ inhibited induction of Jurkat cell aggregation by CD99 mAb MT99/1. It means that the bioactive domain of the CD99 protein expressed on phage particles was preserved.

CD147 molecule is a leukocyte surface molecule, which designated as CD147 molecule at the 6th International Workshop on Human Leukocyte Differentiation Antigen (HLDA workshop) (Stockinger *et al.*, 1997). This molecule also known as M6 Ag (Kasinrerk *et al,* 1992), extracellular matrix metalloproteinase inducer (EMMPRIN) (Biswas *et al,*. 1995) or basigin (Miyauchi *et al.*, 1991). It is a glycoprotein of type 1 transmembrane protein with a molecular weight of 50-60 kDa. CD147 is widely expressed on hemopoietic and

non-hemopoietic cells. It is strongly up-regulated on T cells upon activation, indicating a function in T cell biology (Kasinrerk *et al.,* 1992, Stockinger *et al.,* 1997). However, the function of CD147 molecule is so far unclear. Thus, production of this molecule is a key to trace the ligand-receptor of CD147 molecule.

In an attempt to search the ligand of CD147, we decided to generate phage expressing an extracellular domain of CD147. This phage expressing CD147 will can be used to search for its counter-receptor on various cell types. For this objective, the expressed CD147 fragment must contain bioactive determinants and has to retain the native-like conformation. As CD147 contains two consecutive disulfide bridges in its extracellular domain, domain 1 (Ig V domain) is formed disulfide bond by Cys_{41}-Cys_{87} and Cys_{126}-Cys_{185} are formed for the domain 2 (Ig C2 domain) (Kasinrerk *et al.,* 1992). In our experiments, the production of CD147 molecule linked to phage particle was performed using two *E. coli* host strains, XL-1 Blue and TG-1. The efficiency in expressing phage carrying the proper conformation of CD147 of the two *E. coli* host strains was compared. By sandwich ELISA, phage generated from XL-1 Blue and TG-1 host strains reacted with the CD147 mAb panel in different ways (Figure 3.8). All CD147 mAbs used could capture CD147-Φ produced in TG-1. However, only four CD147 mAbs directed to the CD147 were able to bind the CD147- Φ derived from XL-1 Blue. This result suggested that the conformation of the CD147 epitopes displayed on phage particles delivered from TG-1 was more accurate. The influence of some unknown properties of *E. coli* affecting the production of heterologous proteins is commonly found. Duenas and colleagues

(1994) have studied the influence of bacterial host on the secretion of single-chain Fv antibody fragment (scFv), the production of scFv as intracellular fusion protein, and the effect of chaperonin coexpression on intracellular antibody expression. By using bacterial expression system, they found that the strain of bacteria was an important parameter, but the intracellular fusion protein and chaperonins had no effect on secretion of scFv antibody fragment (Duenas *et al.*, 1994). Moreover, Miksch and colleagues (2002) found that the extracellular production of phytase is increased when the expressing vector which transformed into bacterial host contained *kil* gene. (Miksch *et al.*, 2002). However, to the best of our knowledge, no report has been described for this phenomenon in phage display technique. Since the properly structural folding is tremendously significant in using the recombinant phages as probes for discovering a neo ligand-partner, our findings indicating that care must be taken in using different *E. coli* strains for this purpose.

The correct size of CD147- Φ truncated gpIII fusion protein, 38 kDa, was demonstrated by Western immunoblotting. The antigenic determinants recognized by M6-1B9, M6-1D4, M6-1E9 and M6-1F3 mAbs are in non-tertiary structure. In contrast, M6-2F9 and M6-2B1 mAbs react with conformational epitopes.

In previous CD147 functional study, Kasinrerk and colleagues reported the effects of anti-CD147 mAbs in inducing homotypic cell aggregation of U937. Interestingly, not all of the mAbs tested showed that effect. The mAbs M6-1F3 and M6-2F9 were found to induce U937 homotypic cell aggregation, whereas M6-1E9 was not. They subsequently discovered that the mechanism was depended on

LFA-1/ICAM-1 pathway (Kasinrerk *et al.*, 1999) and the signaling was through protein kinases (Khunkeawla *et al.*, 2001). In addition, mAbs M6-1B9 and M6-1E9 inhibited CD3 inducing T cell proliferation (unpublished observations). From these findings together with the results of epitope mapping (Table 3.1), topographic information of CD147 bioactive epitopes on the CD147-Φ was predicted (Figure 3.10). Since mAbs M6-1B9 and M6-1E9 showed similar result in inhibition of T cell proliferation as well as competition of each other in the epitope mapping experiment, we proposed that the epitopes recognized by these mAbs are contiguous. In contrast, mAb M6-2F9 inhibit T cell proliferation and did not block the binding of mAb M6-1B9 and M6-1E9. The epitope of mAb M6-2F9, therefore, does not overlap or associate with the epitopes recognized by mAbs M6-1B9 and M6-1E9.

The binding of mAb M6-1F3 was interfered with all tested mAbs together with that M6-1F3 could also induce homotypic cell aggregation; the epitope of M6-1F3 was therefore predicted to overlap with other mAbs. We observed that the occupation of mAbs M6-1B9, M6-1E9 and M6-2F9 obstructed the binding of mAb M6-1F3, however, mAb M6-1F3 did not influence the binding of any mAbs tested. This finding may be explained by conformational change after mAbs M6-1B9, M6-1E9 and M6-2F9 interacted with their epitopes. Consequently, other parts of CD147 hampered the epitope recognized by mAb M6-1F3. This phenomenon is regarded as allosteric effect which was reported in certain studies (Davies and Cohen, 1996; Towbin *et al.*, 1996 and Aguilar *et al.*, 2000). The schematic diagram for explaining this phenomenon was simplified in Figure 4.1. In contrast, binding of mAb M6-1F3 to its epitope could not induce the

conformational change of CD147 structure. Another possibility, which could not be excluded, was the affinity difference of the CD147 mAbs. The binding affinity of mAb M6-1F3 may be less effective than other CD147 mAbs, thus it could not block the binding of mAbs M6-1B9, M6-1E9 and M6-2F9 to their epitopes.

In addition, the CD147 displayed on phage particle by *E. coli* is in nonglycosylated form. Thus, it adds more information that all CD147 mAbs used in this study do not require the induction of sugar for recognizing their epitopes. Recently, the deglycosylated CD147 was used for distinguishing a CD147 agonist from an antagonist (Sun and Hemler, 2001). In their experiment, the HT1080 was treated by tunicamycin to prepare the deglycosylated CD147 molecule. However, the result of western immunoblotting demonstrated that tunicamycin did not completely inhibit the process of glycosylation in CD147 molecule. The purified deglycosylated form was used for coincubation with dermal fibroblasts and showed the reduction effect on MMP-1 production. Nevertheless, as the glycosylated form was contaminated in the preparation, the interpretation may need to be confirmed. To establish a more precise experiment, the nonglycosylated phage constructed in this study can be substituted for the deglycosylated CD147 produced in tunicamycin-treated HT1080 cell.

In our study achieved the generation of CD147-Φ and emphasized the necessity of selecting a suitable *E. coli* host strain for proper folding of the displayed molecule. However, there is no common rule applying for each displayed protein. Kurokawa and colleagues analyzed the effects of Dsb overexpression on production of horseradish peroxidase (HRP) isozyme C that contains complex disulfide bonds and tends to aggregate when produced in *E.*

coli. When transported to the periplasm, HRP was unstable but was markedly stabilized upon simultaneous overexpression of the set of Dsb proteins (DsbABCD). Whereas total HRP production increased several fold upon overexpression of at least disulfide-bonded isomerase DsbC, maximum transport of HRP to the periplasm seemed to require overexpression of all DsbABCD proteins, suggesting that excess Dsb proteins exert synergistic effects in assisting folding and transport of HRP. A novel expression strategy for obtaining functional recombinant protein from *E. coli* by co-expression of DsbABCD in periplasm is supposed to overcome this hurdle (Kurokawa *et al.*, 2000). In addition, the relationship between epitope location and bioactive domain was demonstrated by a conventional method. The CD147-Φ will be considered as a screening tool for finding its binding partners on the target cells.

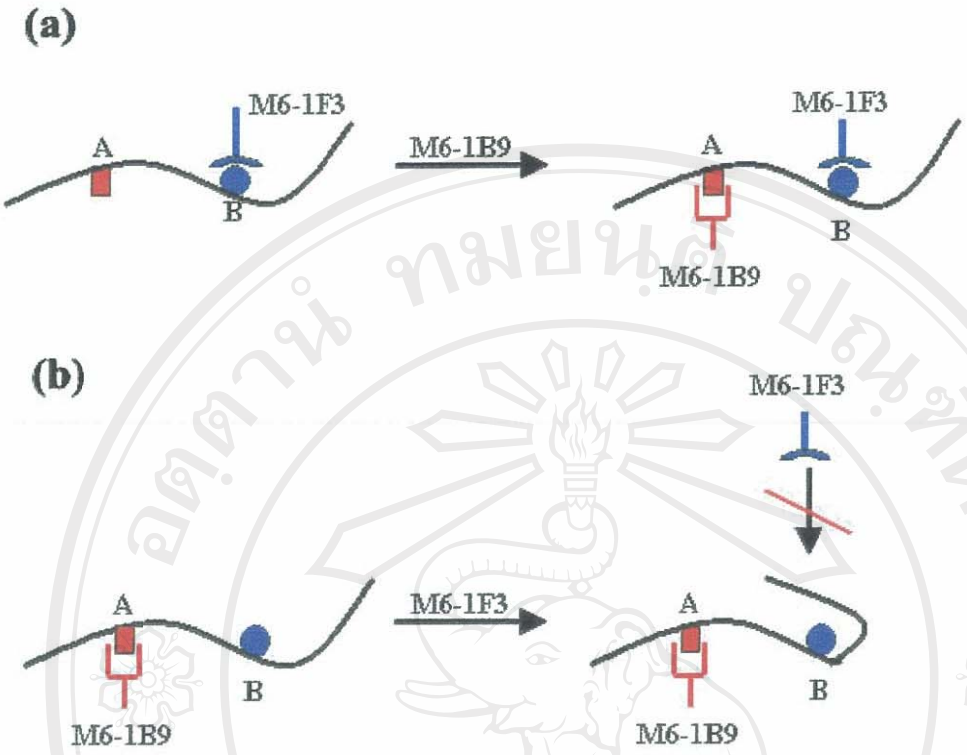

Figure 4.1. Schematic illustration of the allosteric effect of antibody-antigen interaction on CD147 molecule. (a) Binding of M6-1F3 to epitope B did not induce an allosteric effect on the recognition of M6-1B9 (b) Binding of M6-1B9 to epitope A, however, might cause the conformational change in CD147 molecule which hindered the epitope B. Consequently, M6-1F3 was not able to bind to the CD147 molecule.