APPENDIX A

Reagent preparations

1. **10X Tris-acetate/EDTA electrophoresis buffer (TAE)**

Tris-base	**48.40**	**gm**
Glacial acetic acid	**11.42**	**ml**
0.5M EDTA (pH 8.0)	**20**	**ml**

Dissolved all ingredients in distilled water and filled up to 1,000 ml.

Sterilized by autoclave and kept at 4 °C.

2. **1% agarose gel**

Agarose gel	**0.5**	**gm**
1X TAE buffer	**50**	**ml**

Melted by microwave oven.

3. **Ethidium bromide working solution (10 mg/ml)**

Ethidium bromide	**1.0**	**gm**
Distilled water	**100**	**ml**

Kept in the dark bottle and stored at room temperature

4. 6X gel loading buffer

Bromphenol blue	**2.5**	**µl**
Glycerol	**300**	**µl**
Distilled water	**697.5**	**µl**

Mix thoroughly and kept at -20 °C

5. 1 mM Phosphate buffer saline (1X PBS) pH 7.2

NaCl	**8.00**	**gm**
KCl	**0.20**	**gm**
Na_2HPO_4	**1.15**	**gm**
KH_2PO_4	**0.20**	**gm**

Dissolved all ingredient in distilled water and filled up to 900 ml

Adjusted the pH to 7.2 with 1N HCl or 1N NaOH.

Added distilled water to adjust the volume to 1000 ml and stored at 4 °C

6. Reagents for using in SDS-PAGE and Western immunoblotting

6.1 1.5 M Tris-HCl pH 8.8

Tris-base	**18.15**	**gm**
Deionized DW	**75**	**ml**

Adjusted the pH to 8.8 with concentrated HCl

Adjusted the volume to 100 ml with deionized distilled water and stored at 4 °C.

6.2 0.5 M Tris-HCl pH 6.8

Tris-base	**6.0**	**gm**
Deionized distilled water	**80**	**ml**

Adjusted the pH to 6.8 with concentrated HCl

Adjusted the volume to 100 ml with deionized distilled water and stored at 4 °C.

6.3 Running buffer

Tris-base	**1.51**	**gm**
Glycine	**7.20**	**gm**
Sodium dodesyl sulphate	**0.50**	**gm**
Distilled water to	**500**	**ml**

Stored at 4 °C.

6.4 Blotting buffer

Tris-base	**3.03**	**gm**
Glycine	**14.41**	**gm**
Sodium dodesyl sulphate	**0.5**	**gm**
Dissolved all gradients with DW	**700**	**ml**
Methanol	**200**	**ml**
Adjusted the volume to	**1,000**	**ml**

Stored at 4 °C.

6.5 30% Stock Acrylamide

Acrylamide	**15**	**gm**
Bis-acrylamide	**0.5**	**gm**
Distilled water to	**50**	**ml**

Kept at 4 °C.

6.6 Copolymerization of 4% stacking gel (5 ml)

Stock acrylamide 30%	**0.665**	**ml**
0.5 M Tris-HCl pH 6.8	**1.25**	**ml**
SDS 10%	**0.05**	**ml**
Distilled water	**3.01**	**ml**
Ammonium per sulfate 10%	**0.1**	**ml**
TEMED	**0.01**	**ml**

6.7 Copolymerization of 12% Separatinging gel (10 ml)

Stock acrylamide 30%	**4.0**	**ml**
1.5 M Tris-HCl pH 8.8	**2.5**	**ml**
SDS 10%	**0.1**	**ml**
Distilled water	**3.3**	**ml**
Ammonium per sulfate 10%	**0.1**	**ml**
TEMED	**0.01**	**ml**

7. Reagents for using in ELISA

7.1 Washing buffer (500 ml)

1 mM PBS	500	ml
Tween 20	0.25	ml

Mix thoroughly and stored at room temperature.

7.2 Carbonate/bicarnate coating buffer

Na_2CO_3	1.59	gm
$NaHCO_3$	2.93	gm
NaN_3	0.20	gm
Distilled water to	1,000	ml

Kept at 4 °C

8. Reagents for using in plasmid mini-prep.

8.1 3 M Sodium Acetate pH 7.0

Sodium Acetate $3H_2O$	40.8	gm

Adjust pH to 7.0 with NaOH/HCl

Distilled water to	100	ml

Stored at 4 °C

8.2 Potassium Acetate

Potassium Acetate	29.4	gm
Glacial acetic acid	11.5	ml
Distilled water to	100	ml

Stored at 4 °C

8.3 10 M NaOH

NaOH	**200**	**gm**
Distilled water to	**500**	**ml**

Stored at 4 °C

8.4 10% SDS

SDS	**5**	**gm**
Distilled water to	**50**	**ml**

Stored at room temperature.

8.5 7.5 M Ammonium Acetate

Ammonium Acetate	**57.8**	**gm**
Distilled water to	**100**	**ml**

Stored at 4 °C

8.6 1 M glucose buffer

D-glucose	**18.02**	**gm**
Distilled water to	**100**	**ml**

Autoclave and keep at 4 °C

8.7 0.5 M EDTA pH 8.0

EDTA	**37.22**	**gm**

Distilled water	**100**	**ml**

Adjust pH to 8.0 with Conc. HCl

Distilled water to 200 ml

Kept at 4 °C

8.8 1 M Tris pH 8.0

Tris-Base	**24.22**	**gm**
Distilled water	**180**	**ml**

Adjust pH to 8.0 with Conc. HCl

Distilled water to	**200**	**ml**

Autoclave and kept at 4 °C

8.9 10X GLUCOMIX

1 M glucose buffer	**50**	**ml**
0.5 M EDTA pH 8.0	**20**	**ml**
1 M Tris pH 8.0	**25**	**ml**
Distilled water	**5**	**ml**

Autoclave and kept at 4 °C

8.10 1X glucomix-lysozyme solution

10X GLUCOMIX	**300**	**µl**
lysozyme stock (50 mg/ml in distilled water)	**300**	**µl**
Distilled water	**2.4**	**ml**

Stored at 4 °C for 7 days.

9. Media for bacterial culture

9.1 Super Broth

Tryptone	**3.0**	**gm**
Yeast extract	**2.0**	**gm**
Morpholinepropanesulphonic acid (MOPS)	**1.0**	**gm**
Distilled water to	**100**	**ml**

Autoclave and kept at 4 °C

9.2 2xTY Broth

Tryptone	**1.6**	**gm**
Yeast extract	**1.0**	**gm**
Sodium Chloride	**0.5**	**gm**
Distilled water to	**100**	**ml**

Autoclave and kept at 4 °C

APPENDIX B

List of publication

1. Tayapiwatana C., Arooncharus P. and Kasinrerk W. Displaying and epitope mapping of CD147 on VCSM13 phages: influence of *Escherichia coli* strains. Journal of Immunological Methods. 2003; 281: 177-185.

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Recombinant Technology

Displaying and epitope mapping of CD147 on VCSM13 phages: influence of *Escherichia coli* strains

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Abstract

The external domain of a human leukocyte surface molecule, CD147 was displayed on the surface of phage. Two *Escherichia coli* laboratory strains, XL-1 Blue and TG-1, were chosen to separately propagate the recombinant phages. By sandwich enzyme linked immunosorbent assay (ELISA), CD147 on phage particles were individually captured by six CD147 mAbs and subsequently detected by anti-M13 conjugated HRP. All mAbs specifically bound the CD147 on phage particles derived from TG-1. On the contrary, only four of them could recognize the CD147 on phages produced by XL-1 Blue. The results indicate that the environment in the TG-1 periplasm is more appropriate than that of XL-1 Blue for promoting the suitable folding of CD147. This finding emphasizes the importance of selecting the appropriate *E. coli* host for display of a complex protein. The epitopes of CD147 displayed on the phage were further mapped by competitive inhibition ELISA, which is a reliable and economical method. Certain clusters of mAb recognition areas were identified and will provide valuable information for the discovery of the ligand for CD147.

Keywords: CD147; Monoclonal antibodies; Phage display; Epitope mapping; Periplasm

1. Introduction

Expression of heterologous proteins in *Escherichia coli* has long been an essential tool in the study of the structure and function of proteins, in part because of the ease and low cost of manipulation and production. However, not every heterologous protein can be successfully produced in this prokaryotic host, since most recombinant heterologous proteins tend to aggregate, hampering their activity and antigenicity. The periplasm can be a more suitable environment for expression of soluble complex proteins, due to its resemblance to the endoplasmic reticulum of eukaryotic cells (Glockshuber et al., 1992). Compared with the cytoplasm, the periplasm has an oxidizing environment, which promotes better disulfide bond for-

Abbreviations: T.U., transforming unit; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RBS, ribosome binding site; *Omp*A, signal sequence of the outer membranes protein A of *E. coli*.

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mation. The phage display technique, which relies on periplasmic expression of the displayed proteins, has been used successfully for producing various recombinant proteins. The binding activity of ScFv (Andris-Widhopf et al., 2000), antigenicity of tissue plasminogen activator (Manosroi et al., 2001) and bioactive domain of CD99 (Tayapiwatana and Kasinrerk, 2002) are retained.

CD147 is a broadly expressed leukocyte surface molecule. Since the first identification of CD147 (or M6) characteristics (Miyauchi et al., 1991; Kasinrerk et al., 1992), the only CD147-ligand reported was secreted cyclophilin A and B (Yurchenko et al., 2001, 2002). No cell surface ligand-partner of CD147 has been clearly shown. Recently, we reported the effects of CD147 mAbs in inducing homotypic cell aggregation of U937 cells. Interestingly, not all of the mAbs tested bound to the bioactive domains of CD147. We subsequently discovered that the mechanism was depended on the LFA-1/ICAM-1 pathway (Kasinrerk et al., 1999) and the signaling was accomplished through protein kinases (Khunkeawla et al., 2001). It is thus of interesting to determine the ligand for CD147.

Epitope mapping with mAb can provide useful information about the bioactive domains of molecule. Several techniques may be used for epitope characterization, and two have been reported for CD147: the epitope map of a soluble CD147-Fc fusion protein produced from transfected COS cells has been evaluated by BIAcore biosensor, which in principle is accurate but extremely expensive (Koch et al., 1999). More recently, the epitope mapping of CD147 mAbs was analyzed in our laboratory by a fluorescence inhibition technique (unpublished observations). The method is reliable but the eukaryotic expression system is time consuming and requires sophisticated processing. In addition, the competitive mAbs must be labeled with fluorescein dye, which is labour-intensive.

In the present study, we generated phage-displayed CD147 (CD147-ϕ) and mapped its epitopes with defined CD147 mAbs (Kasinrerk et al., 1999; Khunkeawla et al., 2001) by competitive inhibition ELISA. Practically, a number of *E. coli* F^+ strains, e.g. TG-1 (Schlebusch et al., 1997), XL-1 Blue (Lekkerkerker and Logtenberg, 1999), SS320 (Sidhu et al., 2000) and JM109 (Rondot et al., 2001) have been used by various groups for displaying recombinant molecules. The choice of an *E. coli* host strain has been reported as one of the important parameters for producing high level expression of functional heterologous proteins (Friehs and Reardon, 1993; Dueñas et al., 1994; Balbás, 2001). In this study, the efficiency of two *E. coli* laboratory strains, XL-1 Blue and TG-1, in synthesizing the properly folded CD147 on phage particles was evaluated.

2. Materials and methods

2.1. E. coli strains and primers

Two *E. coli* strains, TG-1 {*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F′ [*traD*36*proAB*+, *lac*I^q *lac*ZΔM15]} (kindly provided by Dr. A.D. Griffiths, MRC, Cambridge, UK) and XL-1 Blue {*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*F′ [*proAB*$^+$, *lac*I^q *lac*ZΔM15 Tn10 (tet^r)]} (Stratagene, La Jolla, CA), were used as hosts for the production of phages displaying the CD147 molecule.

Each primer was synthesized with 5′-overhangs containing a *Sfi*I restriction site (small letters): CD147ExF [5′-GAG GAG GAG GTg gcc cag gcg gcc GCT GCC GGC ACA GTC TTC-3′] and CD147ExR [5′-GAG GAG GAG CTg gcc ggc ctg gcc GTG GCT GCG CAC GCG GAG-3′]. They were suitable for annealing the ectodomain of the human CD147 gene from the mammalian expression vector, pCDM8-CD147 (Kasinrerk et al., 1992, 2002), and gave the correct orientation of CD147 gene which was inserted into pComb3HSS phagemid vector, kindly provided by Dr. Carlos F. Barbas, (Scripps Institute, CA).

2.2. CD147 gene amplification by PCR

The external domain of the CD147 gene was amplified using pCDM8-CD147 as a template. Briefly, 50 ng of template was annealed with 1 μg of each primer in 100 μl of a PCR mixture containing 2.5 U of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The amplification condition included a jump start at 85 °C for 4 min and followed by the three cycles of PCR amplification: denaturation at 95 °C for 50 s, annealing at 42 °C for 50 s

and extension at 72 °C for 1.5 min. After 35 amplification cycles, the mixture was incubated at 72 °C for 10 min. Gel electrophoresis was performed to analyze the molecular weight of the PCR product. The amplified product was purified by QIAquick PCR purification Kit (QIAGEN, Hilden, Germany) and cleaved with *Hae*II.

2.3. Construction of phagemid expressing CD147

The phagemid expressing CD147 was constructed by inserting the *Sfi*I-digested ectodomain of CD147 gene into the *Sfi*I-digested pComb3HSS phagemid vector. Fifty nanograms of CD147 amplified product was treated with 1 U of *Sfi*I (Roche Molecular Biochemicals), while 100 ng of pComb3HSS was treated with 5 U of the same enzyme and incubated at 50 °C for 18 h. After purification, the ligation step was performed by adding 1 U of T_4 ligase enzyme (Roche Molecular Biochemicals) into a mixture containing 100 ng of vector and 50 ng of insert. The reaction mixture was subsequently incubated at 4 °C for 16 h. The ligated product was named pComb3H-CD147.

2.4. Bacterial cell transformation

The ligated product, pComb3H-CD147, was transformed into $CaCl_2$ competent *E. coli* XL-1 Blue or TG-1. After culture for 3 h in antibiotic-free LB, the transformed cell pellet was harvested by spinning down at 1100 g, 25 °C for 10 min. The pellet was resuspended in 500 μl of the same medium and plated on LB agar containing ampicillin (100 μg/ml) and cultured overnight at 37 °C. The ampicillin resistant colonies were selected for plasmid miniprep (QIAGEN). Restriction fragment analysis of the purified plasmid was performed using *Sfi*I. Finally, the PCR amplified product was checked for an insert in the purified plasmid as described above.

2.5. Preparation of phage-displayed CD147

For displaying CD147 on filamentous phages, 10 ml of XL-1 Blue bacteria transformed with pComb3H-CD147 was precultured at 37 °C in super broth (3%[wt/vol] tryptone, 2%[wt/vol] yeast extract, and 1%[wt/vol] morpholinepropanesulphonic acid [MOPS]) containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml). When an OD_{600} of 1.5 was reached, the bacteria were transferred to 100 ml of the same medium. Two hours later, the 10^{12} transforming unit (t.u.) of VCSM13 helper phage (Stratagene) was added and cultured for another 3 h. Subsequently, kanamycin (70 μg/ml) was added to the culture, which was continuously shaken at 180 rpm for 18 h at 37 °C. The bacteriophages were harvested by precipitation with PEG 8000 as described previously (Tayapiwatana and Kasinrerk, 2002). Finally, the phages were reconstituted with 0.15 M PBS pH 7.2 and stored at − 70 °C. This protocol was regarded as standard growth condition of XL-1 Blue host.

The pComb3H-CD147 plasmid was transformed into TG-1 strain precultured in 2 × TY broth (1.6% [wt/vol] tryptone, 1%[wt/vol] yeast extract, and 0.5%[wt/vol] sodium chloride) containing ampicillin (100 μg/ml) until an OD_{600} of 0.8 was reached. The precultured bacteria were subsequently propagated in 100 ml of the same medium containing 2 ml of 50% glucose. After 2 h, the 30 ml of culture was infected with 2.4 ml of 10^{12} t.u. of the VCSM13 helper phage and kept at 37 °C without shaking for 30 min. Phage-infected TG-1 was spun down at 1100 g, 4 °C for 10 min. The pellet was reconstituted with 30 ml of 2 × TY broth containing ampicillin (100 μg/ml) and kanamycin (70 μg/ml). Fifteen milliliters of culture was resuspended in 250 ml of the same medium and shaken at 180 rpm for 18 h at 37 °C. The procedures for harvesting and storing the recombinant phages were performed as above. This protocol was regarded as standard growth condition of TG-1 host.

To evaluate the influence of growth conditions, displaying of CD147 on phage particle in TG-1 host was performed under the standard growth condition used for XL-1 Blue host but tetracycline was omitted. CD147-ϕ was conversely assembled in XL-1 Blue host with TG-1 standard growth condition.

2.6. Immunoassay for phage-displayed CD147 by ELISA

Microtiter plates (NUNC, Roskilde, Denmark) was coated with 50 μl of 10 μg/ml CD147 mAbs (M6-1B9; IgG_3, M6-2B1; IgM, M6-1D4; IgM, M6-1E9; IgG_{2a}, M6-1F3; IgM, and M6-2F9; IgM) (Kasinrerk et al., 1999; Khunkeawla et al., 2001) in carbonate/

bicarbonate buffer pH 9.6 for 2 h at room temperature. The plate was then blocked with 2% skimmed milk in 0.15 M PBS pH 7.2 for 1 h at room temperature. The wells were washed four times with 0.05% Tween-20 in 0.15 M PBS pH 7.2 and 10^7 t.u. of recombinant phages were added and the mixture incubated for 1 h at room temperature. The unbound phages were washed out and detection of bound phage was performed using peroxidase-labeled sheep anti-M13 antibodies (Amersham Biosciences, Buckinghamshire, UK). Subsequently, peroxidase activity was determined by treatment with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and measured the optical density (OD) measured at 450 nm after adding 1 M H_2SO_4 to stop the reaction. MT54 mAb specific for CD54 (Moonsom et al., 2001) was used as an antibody control in the ELISA system.

2.7. SDS-PAGE and Western immunoblotting

Phage-expressing CD147 protein were diluted in 5 × non-reducing buffer (3.7%[wt/vol] Tris–HCl, pH 6.8, 5%[wt/vol] sodium dodecyl sulfate, 50%[vol/vol] glycerol) and heat-denatured for 5 min before loading to a 12% separating gel for SDS-PAGE. The separated proteins were blotted to a nitrocellulose membrane. Blocking was performed for 2 h at room temperature with 5% skimmed milk in 0.15 M PBS pH 7.2 and further incubated with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) for 1 h. The membrane was washed three times with 0.05% Tween 20 in 0.15 M PBS pH 7.2 and then incubated with peroxidase-labeled sheep anti-mouse immunoglobulins (DAKO Diagnostica, Hamburg, Germany) diluted in 5% skimmed milk in 0.15 M PBS pH 7.2 for 1 h. Unbound conjugate was washed out three times with 0.05% Tween 20 in 0.15 M PBS pH 7.2 and once with 0.15 M PBS pH 7.2; the specific bands were visualized using a chemiluminescent substrate detection system (Pierce, Rockford, IL).

2.8. Epitope mapping

Epitope mapping was carried out by competitive inhibition ELISA. Fifty microliters of 10 μg/ml CD147 mAbs (M6-1B9, M6-1E9, M6-1F3 and M6-2F9) in carbonate/bicarbonate buffer pH 9.6 was individually absorbed on a solid phase of 96-well plates for 2 h at room temperature. These coated mAbs are referred as catcher. Any nonspecific binding sites were blocked with 2% skimmed milk in 0.15 M PBS pH 7.2. During the blocking period, 10^7 t.u. of CD147-phage (CD147-ϕ) were separately pre-incubated with the same panel of 500 ng of CD147 mAbs which were termed competitors. After the washing step, the pre-incubated CD147-ϕ/CD147 mAbs were added into the CD147 mAbs-coated wells and incubated for 1 h at room temperature. The bound phages were detected by incubating for 1 h at room temperature with peroxidase-labeled sheep anti-M13 antibodies (Amersham Biosciences). After washing, the TMB substrate was added and the reaction was stopped with 1 M H_2SO_4. The reaction of competitive inhibition ELISA was detected at wavelength 450 nm and compared with the OD of non-competitor wells. The cut off value of the inhibition was taken 35% reduction of absorbance units in competitive wells in comparison with the non-competitor wells.

3. Results

3.1. Construction of CD147 phagemid

The ectodomain gene of CD147 in vector pCDM8-CD147 was amplified by PCR using primers CD147ExFw and CD147ExRev. The PCR product containing the double *Sfi*I restriction sites with molecular weight of 552 bp was demonstrated by agarose gel electrophoresis (data not shown). The amplified CD147 gene was sub-cloned into the phagemid-expressing vector, pComb3HSS, in the correct reading frame. The engineered phagemid bearing CD147 ectodomain gene, flanked upstream by *Omp*A signal sequence and downstream by gpIII (Fig. 1), was named pComb3H-CD147.

3.2. Detection of phage-displayed CD147 from different E. coli strains

Recombinant bacteriophages were produced by infecting the pComb3H-CD147-transformed *E. coli* with VCSM13 helper phage. During the assembly of progeny viruses, the CD147-gpIII fusion proteins were concomitantly incorporated into phage particles. To detect phage carrying CD147 molecules released

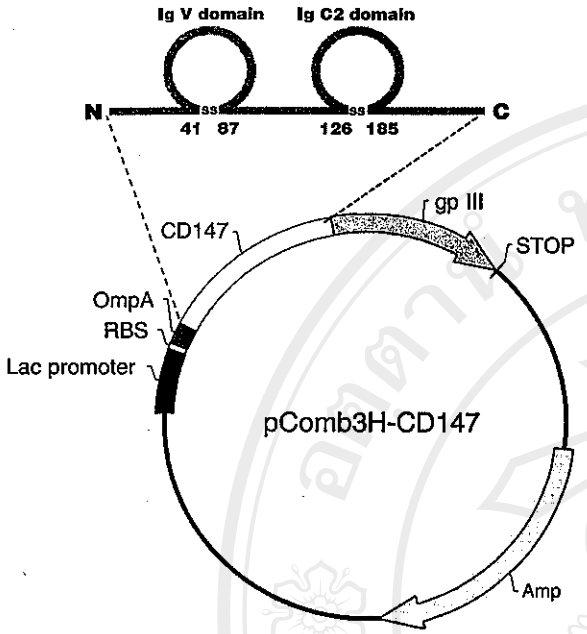

Fig. 1. Schematic representation of pComb3H-CD147: Double *Sfi*I-cloning sites where the CD147 ectodomain gene was inserted; the signal sequence (*Omp*A), ribosome binding site (RBS), *lac* promoter and gpIII gene are depicted. STOP represents the stop codon for CD147-gpIII translation. The derived primary structure of CD147 ectodomain containing two immunoglobulin-like domains is shown. The cysteine residuals which form disulfide bridges are labeled.

into culture supernatant, the polystyrene plate was coated with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) for sandwich ELISA. Only four of the six mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-2F9) reacted against CD147-ϕ derived from *E. coli* XL-1 Blue host when culturing in its standard growth condition (Fig. 2). In contrast, the CD147-ϕ produced in *E. coli* TG-1 under its standard growth condition could be recognized by all CD147 mAbs used. No binding was seen to CD54 mAb (MT54)-coated well which was used as a control. None of CD147 mAbs-captured phages expressing the irrelevant protein, CD99 (Tayapiwatana and Kasinrerk, 2002) (data not shown). This indicated the specificity of CD147 mAbs used. Our results demonstrated that different *E. coli* strains produced different conformation of the expressed protein on phage particles. However, this effect may influence from the different growth conditions. To address this question, CD147-ϕ was produced in TG-1 using standard growth condition of XL-1 Blue and vice versa.

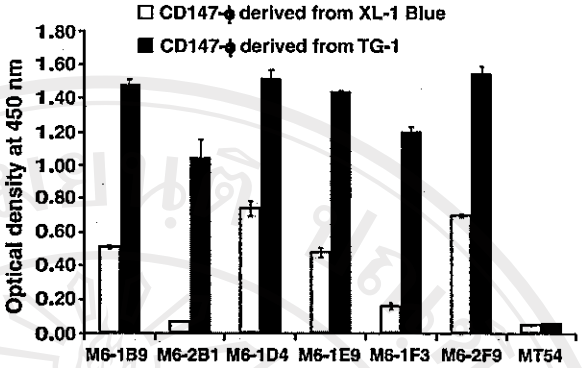

Fig. 2. Comparison of the binding efficiency of CD147-ϕ derived from *E. coli* XL-1 Blue or TG-1 host to the indicated CD147 mAbs by sandwich ELISA. Six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) and one irrelevant CD54 mAb (MT54) were individually immobilized on polystyrene plates. The antibody-bound phages were detected with anti-M13 conjugated HRP. The experiment was performed twice with two preparations of CD147-ϕ from both bacterial strains. The histograms demonstrated the mean value and standard deviation.

The CD147-ϕ produced in TG-1 with the standard growth condition of XL-1 Blue could be recognized by all CD147 mAbs used (Fig. 3). However, the number of CD147-ϕ captured by mAb M6-1F3 was remarkably decreased in comparison to CD147-ϕ produced in

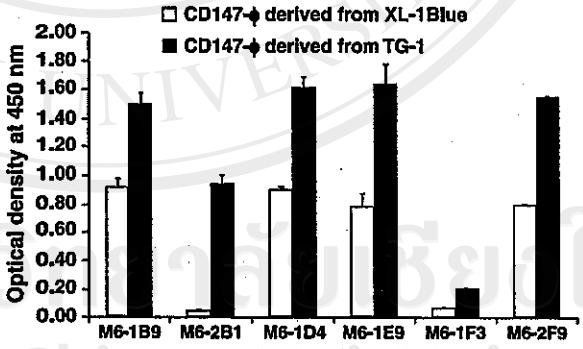

Fig. 3. Influence of growth condition on the folding of CD147 epitopes. CD147-ϕ derived from XL-1 Blue host using the standard growth condition of TG-1 and vice versa were detected with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9). The antibody-bound phages were detected with anti-M13 conjugated HRP. The experiment was performed twice with two preparations of CD147-ϕ from both bacterial strains. The histograms demonstrated the mean value and standard deviation.

TG-1 with its standard growth condition (Fig. 2). CD147 mAbs, M6-2B1 and M6-1F3, could not capture CD147-ϕ derived from XL-1 Blue which was cultured in the standard growth condition of TG-1.

3.3. Western immunoblotting

Protein components of CD147-ϕ generated from *E. coli* TG-1 were separated by SDS-PAGE under non-reducing conditions. The polypeptides were transferred onto a nitrocellulose membrane and subsequently probed with the six CD147 mAbs. An immuno-reactive band located at approximately 38 kDa was obtained with four CD147 mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-1F3) (Fig. 4). This suggests the fusion protein of CD147 ectodomain (20 kDa) and truncated gpIII (18 kDa). The separated polypeptides did not interact with CD54 mAb (MT54), which was used as a negative control. A specific band with molecular weight of 40 kDa was observed when probing with anti-gpIII mAb, demonstrating the presence of the VCSM13 component in the loaded sample.

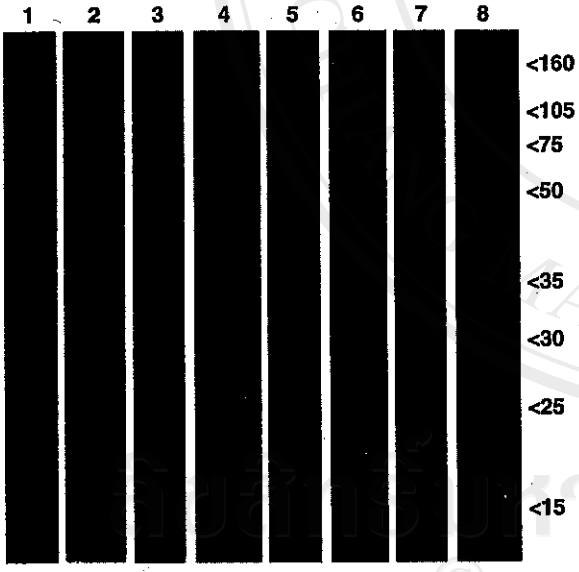

Fig. 4. Western immunoblotting of CD147-ϕ proteins separated by non-reducing SDS-PAGE. Immunological assay was performed by probing with CD147 mAbs; M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9 (lanes 1–6, respectively), CD54 mAb (MT54) (lane 7) or anti-gpIII of VCSM13 mAb (lane 8). The immunoreactive bands were visualized by chemiluminescent substrate detection system. Molecular weight markers in kDa were indicated by arrows.

Table 1
The optical density of competitive inhibition ELISA for epitope mapping of CD147 mAbs

Inhibitor	Catcher			
	M6-1B9	M6-1E9	M6-1F3	M6-2F9
M6-1B9	**1.06**	0.78	0.07	1.69
M6-1E9	1.07	**0.96**	0.06	1.32
M6-1F3	1.63	1.59	**0.22**	1.50
M6-2F9	1.50	1.57	0.07	**1.04**
No inhibitor	1.69	1.64	1.56	1.61

The absorbance units of self-inhibition were designated in bold letter. The absorbance units which showed more than 35% reduction in comparison with no inhibitor were underlined.

3.4. Epitope mapping

Competitive inhibition ELISA was used for epitope mapping analysis of CD147 ectodomain presented on phage particles derived from *E. coli* TG-1. Four CD147 mAbs (M6-1B9, M6-1E9, M6-1F3, and M6-2F9) were used for the epitope mapping. In this experiment, each CD147 mAb, which was used as the inhibitor, was incubated with CD147-ϕ in the soluble phase. The same set of CD147 mAb was separately immobilized on ELISA wells and used as the catcher. The peroxidase-labeled sheep anti-M13 antibodies were used to determine whether CD147 mAb pre-incubated CD147-ϕ was captured on the solid phase by the catcher. If the competitor and catcher bound to the same region on CD147 molecule, CD147-ϕ would not be caught on the solid phase. Self-inhibition was used to indicate maximal inhibition control. The data of competitive inhibition ELISA is shown as absorbance units in Table 1. Each reaction pair of inhibitor and catcher, which gave more than 35% reduction of absorbance unit in comparison with the non-competitor well, was taken as indicating an overlapping epitope. In this experiment, mAbs M6-1B9 and M6-1E9 inhibited each other. MAb M6-2F9 did not hamper the binding of either mAb M6-1B9 or M6-1E9, and vice versa. Binding of mAb M6-1F3 was interfered with by all tested mAbs. In contrast, mAb M6-1F3 did not block the occupation of other mAbs. As a result, the epitopes of extracellular domain CD147 were proposed as falling into four groups (Fig. 4).

. Discussion

Since phage display technology was invented in)85 (Smith, 1985), certain investigations have dem-ıstrated that phage display is a high potential tech-)logy for producing functional recombinant proteins ıppenzeller et al., 2001; An et al., 2002) Recently, e have applied this technique to generate phage :pressing a leukocyte surface molecule, CD99 'ayapiwatana and Kasinrerk, 2002). By this tech-que, the bioactive domain of the CD99 protein :pressed on phage particles was preserved. The 'fects on cellular changes of haematopoietic cell lines / CD99-ϕ, i.e. homotypic cell aggregation, prolifer-ion and apoptosis, suggested the presence of a)unter-receptor (unpublished observations).

In an attempt to characterize the ligand of CD147, e decided to generate phage expressing a fragment of D147. The aim is to use CD147-ϕ to search for its)unter-receptor on various cell types. However, for is objective, the expressed CD147 fragment must)ntain bioactive determinants and has to retain the ıtive-like conformation. As CD147 contains two)nsecutive disulfide bridges in its extracellular do-ain (Kasinrerk et al., 1992), we compared the effi-ency of the two *E. coli* host strains, XL-1 Blue and G-1, in expressing phage carrying the proper confor-ation of CD147. By sandwich ELISA, phage gener-ed from XL-1 Blue and TG-1 host strains reacted ith the CD147 mAb panel in different ways. All D147 mAbs used could capture CD147-ϕ produced TG-1. However, only four CD147 mAbs directed to e CD147 were able to bind the CD147-ϕ derived)m XL-1 Blue. This result suggested that the con-rmation of the CD147 epitopes displayed on phage rticles delivered from TG-1 was more accurate. The fluence of some unknown properties of *E. coli* fecting the production of heterologous proteins is mmonly found (Dueñas et al., 1994; Miksch et al.,)02). However, to the best of our knowledge, no port has been described for this phenomenon in age display technique. Since the properly structural lding is tremendously significant in using the recom-nant phages as probes for discovering a neo ligand-rtner, our findings indicating that care must be taken using different *E. coli* strains for this purpose.

Since the growth condition for XL-1 Blue and TG-were different, we raised a question whether the phenomenon described above is the effect of *E. coli* strains alone. Hence, the standard growth condition of XL-1 Blue was used to produce CD147-ϕ in TG-1 and vice versa. It was found that phage generated from XL-1 Blue and TG-1 host strains in the switched growth conditions reacted with the CD147 mAb panel with pattern almost the same as those obtained from its standard growth conditions. Surprisingly, mAb M6-1F3 reacted much better to CD147-ϕ produced from TG-1 in its standard growth condition than those generated in XL-1 Blue growth condition. This finding suggested that both *E. coli* strain and growth condition are important. A suitable culturing condition and a proper *E. coli* host must be cautiously selected for obtaining the correct conformation of CD147 displayed on phage particle.

The correct size of CD147-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 our previous CD147 functional study, mAbs M6-1F3 and M6-2F9 were found to induce U937 homotypic cell aggregation, whereas M6-1E9 was not (Kasinrerk et al., 1999; 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 1), topographic information of CD147 bioactive epitopes on the CD147-phage was predicted (Fig. 5). Since mAbs M6-1B9 and M6-

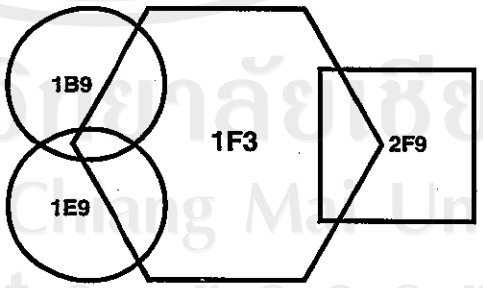

Fig. 5. Topographic illustration of the predicted CD147 bioactive epitopes. Each geometric form represented individual epitope recognized by mAb. The intersections of the polygons indicate the overlapping regions of the different epitopes.

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 could not induce cell aggregation or 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; Aguilar et al., 2000). 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 summary, 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. 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.

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