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ORIGINAL PAPER

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Construction and characterization of phage-displayed leukocyte surface molecule CD99

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Abstract The phage display technique has been described for the production of various recombinant molecules. In the present report, we used this technique to display a leukocyte surface molecule, CD99. PCR subcloning of CD99 cDNA from the mammalian expression vector pCDM8 to the phagemid expression vector pComb3HSS was performed. The resulting phagemid, pComb3H-CD99, was transformed into *Escherichia coli* XL-1 Blue. CD99 was displayed on the phage particles following infection of the transformed *E. coli* with the filamentous phage VCSM13. Using sandwich ELISA, the filamentous phage-displayed CD99 was captured by a CD99 monoclonal antibody (mAb) then detected with anti-M13 conjugated to horseradish peroxidase, confirming that the CD99 molecule was displayed on the phage particles. The CD99-phages inhibited induction of Jurkat cell aggregation by CD99 mAb MT99/1. Proper folding of the displayed CD99 bioactive domain was inferred from this finding. Our results demonstrate that the phage display technique can be applied to the generation of full-length CD99 molecules. The phage carrying this cell surface protein will be useful for identification of its counter receptor or ligand.

Introduction

The filamentous phage display technique, first described by Smith (1985), has been widely used for expression of polypeptides and proteins. Currently, several phagemid vectors are available for different purposes (McCafferty et al. 1994; Crameri and Blaser 1996; Persic et al. 1997). Basically, the recombinant molecules are fused with phage coat proteins, gp3 or gp8, and displayed on the surface of phage particles. The phage display technique delivers the recombinant molecule to the periplasm of *Escherichia coli* with the assistance of signal peptides. Due to the higher oxidizing conditions in comparison to the cytoplasm, the periplasmic environment effectively promotes disulfide bond formation (Becker and Hsiung 1986; Dracheva et al. 1995). Therefore, using phage display technique, the correct conformation of a recombinant protein is obtained. In 1995, Barbas and Wagner constructed the phagemid vector pComb3HSS for delivering a Fab fragment to the periplasmic space of *E. coli* (Barbas and Wagner 1995). Phage-displayed Fab libraries were produced and used to select Fabs specifically interacting with the epitope of interest. Recently, this vector was applied to the production of a tissue plasminogen activator deletion mutant (K2S) that contains nine disulfide bridges (Manosroi et al. 2001). Other complex molecules have also been displayed using this vector (Lasters et al. 1997; Appenzeller et al. 2001; Kurokawa et al. 2002). Production of recombinant protein by phage display technique has the major advantage that the displayed recombinant molecules can be directly and easily harvested from the *E. coli* culture supernatant by PEG precipitation.

Leukocytes express a large number of molecules on their surfaces. These leukocyte surface molecules are important for cell function. Antibodies raised against these molecules have become a major tool in characterizing the structure and function of these surface molecules. In addition to specific antibodies, the isolated cell surface molecules themselves have been broadly used for identification and functional characterization of their counter receptors or specific ligands (Bowen et al. 1996; Vilardell et al. 1998; Martinez-Pomares et al. 1999). However, to prepare these molecules from cell membranes, cumbersome steps of specific cell isolation are

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required. In addition, contamination with undesired proteins is difficult to avoid. To overcome this problem, molecular techniques in mammalian cell expression systems have been employed. The Fc of immunoglobulin was used as a fusion partner of certain CD molecules, e.g., CD31 (Prager et al. 1996) and CD147 (Koch et al. 1999). The fusion protein was then secreted into the culture medium and purified using a protein A column. Although the recombinant proteins obtained have an almost native conformation, mammalian cell expression systems are more expensive and time-consuming in comparison to prokaryotic expression systems. Moreover, the hydrophobic nature of the transmembrane region of the CD molecule makes it impossible to produce the entire length version as a secreted protein. An influence of the transmembrane domain on the conformational structure of the external domain has already been shown (Gaudin et al. 1999). In contrast, lipocalin-1 interacting membrane receptor, a molecule with nine putative transmembrane domains, was successfully expressed using phage display (Wojnar et al. 2001).

In an attempt to produce recombinant leukocyte surface molecules using a prokaryotic expression system, the potential of the phage display technique was evaluated. We demonstrated that the phage display technique could be used to generate phage displayed CD99 molecules. The constructed phages were able to inhibit Jurkat cell aggregation induced by monoclonal antibodies (mAb) against CD99, indicating the presence of a bioactive domain. Our findings suggest that the phage display technique is useful for displaying cell surface molecules when the corresponding cDNA is available.

Materials and methods

Primer design

A pair of primers, CD99MatF 5'-GAGGAGGAGGTGGCCCAGGCGGCCGATGGTGGTTTCGATTTA-3' and CD99MatR 5'-GAGGAGGAGCTGGCCGGCCTGGCCTTTCTCTAAAAGAGTACG-3' (synthesized at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Thailand) were designed to amplify the mature CD99-encoding gene carried by the mammalian expression vector pCDM 8 (Kasinrerk et al. 2000). The primers were designed with *Sfi*I end cloning sites (underlined) to maintain the correct reading frame of the inserted sequence from the ATG to the gpIII gene in the phagemid vector pComb3HSS.

PCR amplification of the CD99 gene

Primers CD99MatF and CD99MatR (1 µg each) together with 50 ng CD99-encoding cDNA (pCDM 8-CD99) template were suspended in a 100 µl PCR mixture. *Taq* polymerase (2.5 U; Roche, Indianapolis, Ind.) was added last to the solution. The titrated amplification condition was initiated with a jump start at 85°C for 4 min, then denaturation at 95°C for 50 s, annealing at 42°C for 50 s, then extension at 72°C for 1.5 min for 35 cycles. The mixture was further incubated at 72°C for 10 min. The amplified product of 537 bp was subsequently purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The correct identity of the purified product was confirmed by restriction enzyme fragment analysis.

Construction of a phagemid expressing CD99

The purified CD99 PCR product and the phagemid pComb3HSS (kindly provided by C.F. Barbas, Scripps Institute, La Jolla, Calif.) (Barbas and Wagner 1995) were digested with *Sfi*I (Roche) to prepare specific cohesive cloning sites. Purified PCR product (4 µg) was digested with 60 U *Sfi*I at 50°C for 18 h; for pComb3HSS, 20 µg phagemid was treated with 100 U *Sfi*I. Digested fragments of the purified PCR products and pComb3HSS (~3,300 bp) were subsequently gel-purified using a QIAquick Gel Extraction Kit (Qiagen). A ligation reaction was performed by introducing 5 U T4 DNA ligase (Roche) to a mixture of 0.7 µg purified *Sfi*I-digested pComb3HSS and 0.9 µg purified *Sfi*I-digested PCR product. Ligation was performed at 30°C for 18 h. The newly constructed phagemid was named pComb3H-CD99.

Transformation of XL-1 Blue

$CaCl_2$ competent *E. coli* XL-1 Blue (200 µl) (Stratagene, La Jolla, Calif.) were transformed with 70 ng ligated product. The transformed cells were propagated by spreading on LB agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline (Sigma, St. Louis, Mo.). After cultivation at 37°C for 18 h, several antibiotic-resistant colonies were selected for plasmid minipreps using the alkaline lysis method. Each purified plasmid was subjected to *Sfi*I restriction site analysis. A transformant harboring a plasmid with the correct *Sfi*I restriction pattern was subsequently propagated for 18 h at 37°C in 100 ml LB broth with antibiotics as above. A plasmid maxiprep was performed using the Qiagen Plasmid Maxi Kit. The purified plasmid was re-examined by *Sfi*I digestion.

Preparation of CD99-ϕ

After transforming XL-1 Blue with pComb3H-CD99, the phage display technique was performed. A clone of pComb3H-CD99-transformed XL-1 Blue was propagated in 10 ml super broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline at 37°C until an OD at 600 nm of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and cultured for another 2 h. The transformed XL-1 Blue were infected with 10^{12} pfu VCSM13 helper phage (Stratagene). After 3 h incubation, kanamycin (final concentration 70 µg/ml) was added to the culture, which was then left shaking (200 rpm) for a further 18 h at 37°C. Bacteriophages harboring CD99 on gpIII (CD99-ϕ) were then precipitated using 4% (w/v) PEG MW 8000 (Sigma) and 3% (w/v) NaCl. Finally, the harvested phage was resuspended in 2 ml phosphate-buffered saline (PBS), pH 7.4.

Immunoassay for CD99-ϕ

CD99-specific mAbs, MT99/1 (IgM isotype) and MT99/3 (Ig2a isotype), were generated in our laboratory (unpublished data, and Kasinrerk et al. 2000). Solid phase was separately coated with 1 µg MT99/1 and MT99/3. The same amount of a CD54 mAb, MT54 (Moonsom et al. 2001), was used as a control. Standard ELISA washing and blocking processes were performed. CD99-ϕ or VCSM13 phages (50 µl; 10^{11} pfu/ml) were added to each mAb-coated well. A suitable dilution of horseradish peroxidase (HRP)-conjugated sheep anti-M13 (Pharmacia, Uppsala, Sweden) was added to each reaction well after the washing step. The 3,3',5,5'-tetramethylbenzidine plus H_2O_2 substrate was added to every well and the reaction was finally stopped with H_2SO_4 solution after a 30-min incubation.

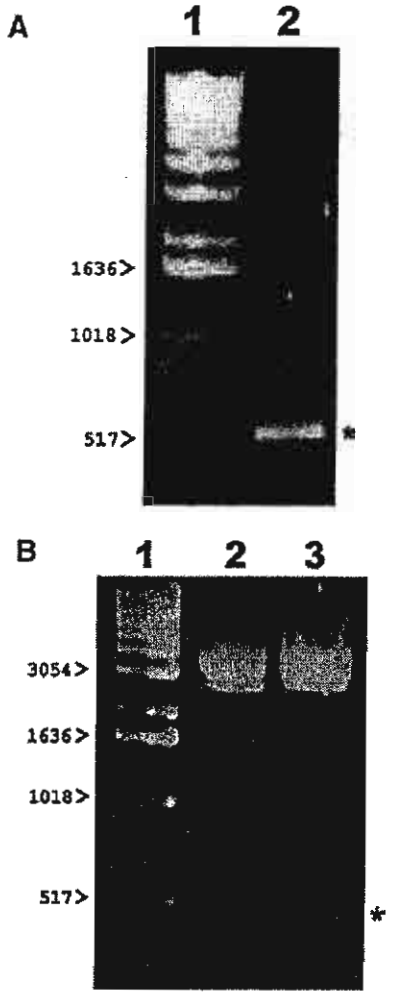

Fig. 1 **a** CD99 gene PCR amplification product from the pCDM 8-CD99 vector using primers CD99MatF and CD99MatR. Lanes: *1* DNA molecular weight marker (Roche), *2* 1 µl amplified product. A single band at 537 bp is depicted (*). **b** Restriction fragment analysis of pComb3-CD99. The constructed pComb3-CD99 was digested with *SfiI* and electrophoresis was performed on a 1% agarose gel. Lanes: *1* DNA molecular weight marker, *2* uncut pComb3H-CD99, *3* *SfiI*-digested pComb3H-CD99; inserted CD99 gene at 489 bp (*)

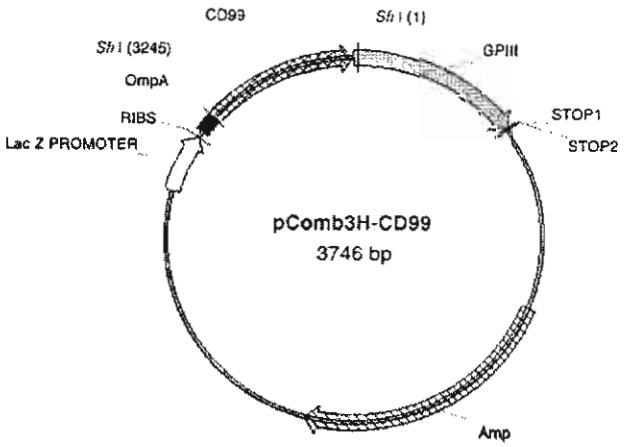

Fig. 2 Map of pComb3H-CD99. The two *SfiI* cloning sites into which the CD99 gene was inserted are indicated. Signal sequence (*OmpA*), ribosome binding site (*RIBS*), *lac* promoter, and *gpIII* gene are also depicted

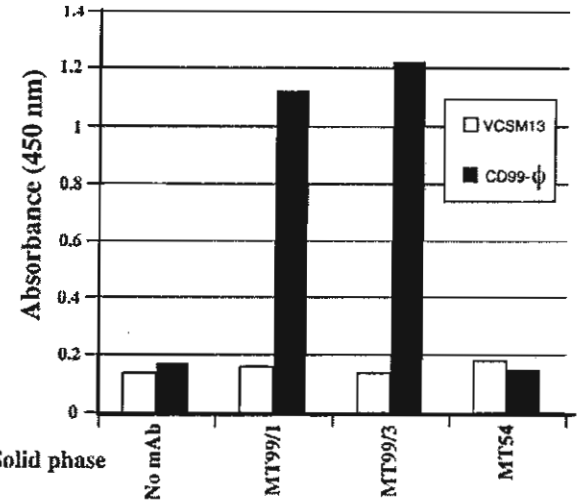

Fig. 3 Sandwich ELISA for the detection of phage bearing CD99. Solid phase was coated with either MT99/1, MT99/3, MT54 or no monoclonal antibody (mAb). VCSM13 was used as a negative control. The bound phage was traced with horseradish peroxidase (HRP)-conjugated sheep anti-M13

Validation of bioactive domain on CD99-ϕ by aggregation inhibition assay

The Jurkat human T-cell line was used as a target for homotypic cell aggregation. After washing three times, 75 µl Jurkat cells (2.5×10^5 cells/ml) were transferred to a 96-well flat-bottomed tissue culture plate (Costar, Cambridge, Mass.). The aggregation base line was obtained by adding 50 µl of 0.15 µg/ml MT99/1 to the well. For the aggregation inhibition assay, 50 µl of 10^{12} pfu/ml CD99-ϕ were preincubated with 50 µl of 0.15 µg/ml MT99/1 for 1 h at 37°C before adding to the Jurkat cells. VCSM13 was used in place of CD99-ϕ as a negative inhibition control system. The final volume of each well was adjusted to 175 µl with culture medium. The culture was then maintained at 37°C in a humidified atmosphere with 5% CO_2 in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. Cell aggregation was monitored every hour for 4 h under an inverted microscope (Olympus, Tokyo, Japan).

Results

Construction of a phagemid expressing CD99

In order to generate phage expressing CD99 molecules, a cDNA encoding CD99 protein cloned in the eukaryotic expression vector pCDM 8 (pCDM 8-CD99) (Kasinrerk et al. 2000) was used. From pCDM 8-CD99, we amplified the mature CD99 gene using primers CD99MatF and

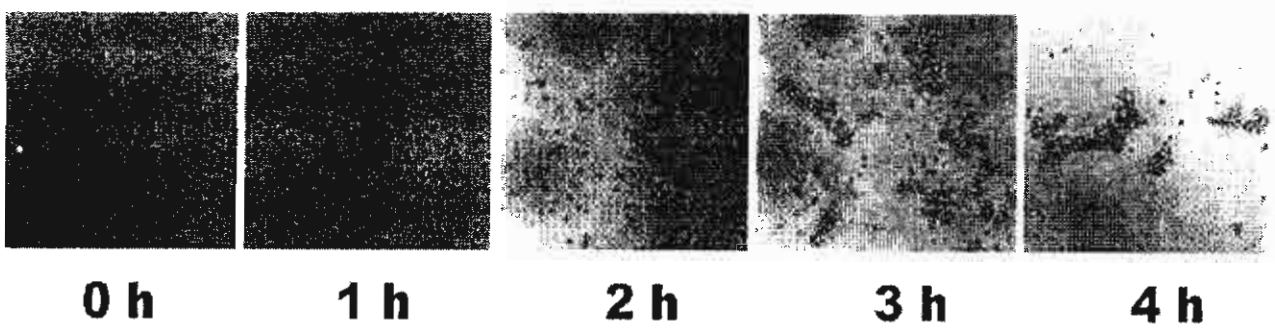

Fig. 4 Induction of Jurkat cell aggregation by MT99/1. Jurkat cells were incubated with MT99/1. Homotypic cell aggregation was monitored under an inverted microscope for 4 h

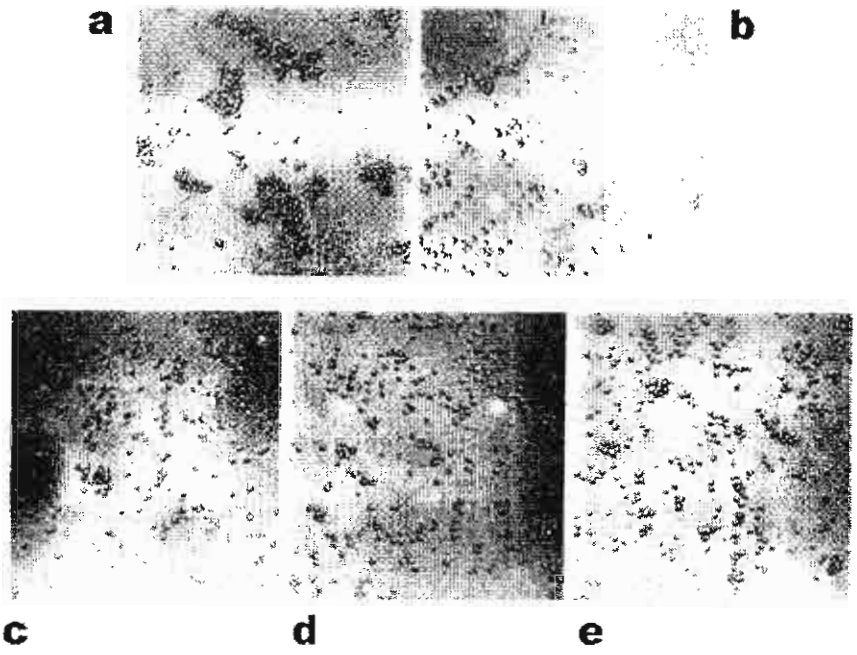

Fig. 5a–e Inhibition of MT99/1-induced Jurkat cell aggregation by CD99-ϕ. Jurkat cells were incubated with MT99/1 preincubated with VCSM13 (a) or CD99-ϕ (b). As controls, Jurkat cells were cultured with VCSM13 (c) or CD99-ϕ (d) alone. Non-induced Jurkat cells were referred as auto-aggregation base-line control (e). The degree of aggregation was observed after 4 h of cultivation

CD99MatR. The amplified product of 537 bp obtained (Fig. 1A) was then inserted into pComb3HSS phagemid in the correct reading frame by means of the *Sfi*I cleavage sites on both the 5' and 3' ends. Thus, a new vector, pComb3H-CD99, harboring the CD99 gene was generated. In this vector, CD99-DNA is flanked upstream by the OmpA signal sequence and downstream by gpIII. The correct insertion of CD99 was verified by restriction analysis with *Sfi*I (Fig. 1B). PCR-analysis using primers CD99MatF and CD99MatR produced a single band of 537 bp. A map of pComb3H-CD99 is shown in Fig. 2.

Generation of phage displaying the CD99 molecule

To produce phage displaying CD99 (CD99-ϕ), VCSM13 filamentous phage was used to infect pComb3H-CD99-transformed *E. coli* XL-1 Blue. Propagation of VCSM13 results in incorporation of the CD99-gpIII fusion protein during the viral packaging process. The recombinant phage particles thus produced were screened for the expression of recombinant CD99 by sandwich ELISA. As shown in Fig. 3, the generated CD99-ϕ specifically bound to both CD99 mAbs (MT99/1 and MT99/3). In contrast, VCSM13 prepared from non-transformed XL-1 Blue was not captured by either CD99 mAb. A negative result was also obtained in wells coated with CD54 mAb MT54, irrespective of the phage type added (Fig. 3). These results suggested that CD99-expressing phage particles had been successfully produced.

CD99-expressing phages carry a bioactive domain

It has been demonstrated that CD99 mAbs induce homotypic Jurkat cell aggregation (Kasinrerk et. al.

2000). In the presence of MT99/1, Jurkat cells started to show homotypic cell aggregation after 1 h incubation and reached maximum aggregation at 4 h incubation (Fig. 4). The induction of cell aggregation by MT99/1 was then used to evaluate the CD99 bioactive domain on CD99-ϕ. As shown in Fig. 5, preincubation of MT99/1 with CD99-ϕ inhibited Jurkat aggregation. In contrast, induction of cell aggregation was not altered after preincubation of MT99/1 with VCSM13. When Jurkat cells were cultured in the presence of VCSM13 or CD99-ϕ alone, very few auto-aggregation foci resulted after 4 h incubation (Fig. 5). The same degree of auto-aggregation degree also appeared in the non-induction Jurkat culture control (Fig. 5). These results indicated that the generated CD99-ϕ carry a properly folded bioactive epitope, which was recognized by MT99/1.

Discussion

The phage display technique has been described for the production of recombinant molecules such as antibodies (Hoogenboom and Chames 2000), tissue plasminogen activator (Manosroi et al. 2001), or collagen-binding protein from *Necator americanus* (Viaene et al. 2001). The conformational structure of the heterologous molecules can be vastly improved as they are delivered to the periplasmic space of *E. coli*, which has higher oxidizing conditions compared to the cytoplasm. In the present report, we genetically engineered a cell surface molecule, CD99, using phage display. The PCR-amplified CD99 cDNA was inserted into *Sfi*I-cleaved pComb3HSS phagemid. The resulting phagemid (pComb3H-CD99) was then used to generate CD99-expressing phages using helper phage VCSM13. Expression of CD99 was demonstrated by sandwich ELISA; CD99-ϕ were recognized by CD99 mAbs MT99/1 and MT99/3. Since an HRP-labeled anti-M13 phage antibody was used as the tracing antibody, the CD99 molecules were manifestly linked to phage particles.

mAbs against CD99 protein produced in our department were previously shown to induce homotypic cell aggregation of Jurkat cells (Kasinrerk et al. 2000). In the present study, the inhibition of Jurkat cell aggregation induced by MT99/1 was used to evaluate the presence of CD99 bioactive domains on CD99-ϕ. The degree of Jurkat cell aggregation was significantly reduced when MT99/1 was preincubated with CD99-ϕ. The CD99 molecule was clearly implicated as the inhibitor since preincubation of MT99/1 with VCSM13 did not obstruct cell aggregation. The inhibition of MT99/1-induced Jurkat aggregation by CD99-ϕ suggested that the generated CD99-ϕ contained a properly folded bioactive domain. This successful preservation of the bioactive domain allows further use of CD99-ϕ in the screening of specific ligands on the leukocyte surface.

Taken together, our findings demonstrate the feasibility of using the phage display technique to display the CD99 molecule. This technique has a high potential to generate phage expressing other leukocyte surface molecules, providing the corresponding cDNA is available. In practical terms, the recombinant phages produced will be useful for identification and functional analysis of the receptors of the molecules of interest. In addition, the recon binant phagemid can be easily switched from a phage display version to a secretory version without subcloning to a new vector, as demonstrated in our recent study (Manosroi et al. 2001). As the defined fermentation conditions allowed protein levels of 100 mg/ml to be obtained, an adequate quantity of soluble molecule of interest can be produced (Manosroi et al. 2002). The soluble protein produced can be used for other immunological studies, e.g., epitope characterization and immunomodulation assays.

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

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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*

Abstract

The external domain of a human leukocyte surface molecule, CD147 was displayed on the surface of phage. Two *E. coli* laboratory strains, XL-1 Blue and TG-1, were chosen to separately propagate the recombinant phages. By sandwich 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 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 *E. coli* has long been as an essential tool in the study of the structure and function of proteins, in part because of the easy 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 formation. 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 the secreted cyclophilin A and B (Yurchenko et al., 2001; Yurchenko et al., 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 were 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 and mapped its epitopes with the 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; Balbas, 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 hsdΔ5 thiΔ(lac-proAB)* F' [*traD36proAB+*, *lac*I^q *lacZ*ΔM15]} (kindly provided by Dr. A.D. Griffiths, MRC Cambridge, UK) and XL-1 Blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F' [*proAB*$^+$, *lac*I^q *lacZ*ΔM15 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., 2002), and gave the correct orientation of CD147 gene which 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 3 cycles of PCR amplification: denaturation at 95 °C for 50 sec, annealing at 42 °C for 50 sec 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. 50 ng 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 1,100 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} 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.

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 were infected with 2.4 ml of 10^{12} t.u. of the VCSM13 helper phage and kept at 37°C without shaking for 30min. Phage-infected TG-1 was spun down at 1,100 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 ml 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.

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 phage was 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 Immunobloting

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 1h. 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 GmbH, 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 μl of 10 μg/ml CD147 mAbs (M6-1B9, M6-1E9, M6-1F3 and M6-2F9) in carbonate/bicarbonate buffer pH 9.6 were 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 subjected as 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 phage was 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 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 (Fig. 2). In contrast, the CD147-ϕ produced in *E. coli* TG-1 could be recognized by all CD147 mAbs used. No binding was seen to CD54 mAb (MT54) coated well was also used as a control. None of CD147 mAbs

captured phages carrying the irrelevant protein, CD99 (Tayapiwatana and Kasinrerk, 2002) (data not shown). This indicated the specificity of CD147 mAbs used.

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. 3). 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.

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 were 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 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).

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 (Appenzeller et al., 2001; An et al., 2002) Recently, we have applied this technique to generate phage expressing a leukocyte surface molecule, CD99 (Tayapiwatana and Kasinrerk, 2002). By this technique, the bioactive domain of the CD99 protein expressed on phage particles was preserved. The effects on cellular changes of haematopoietic cell lines by CD99-ϕ i.e. homotypic cell aggregation, proliferation and apoptosis, suggested the presence of a counter-receptor (unpublished observations).

In an attempt to characterize the ligand of CD147, we decided to generate phage expressing a fragment of CD147. The aim is to use CD147-ϕ to search for its counter-receptor on various cell types. However, 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 (Kasinrerk et al., 1992), we compared the efficiency of the two *E. coli* host strains, XL-1 Blue and TG-1, in expressing phage carrying the proper conformation of CD147. By sandwich ELISA, phage generated from XL-1 Blue and TG-1 host strains reacted with the CD147 mAb panel in different ways. 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 (Dueñas et al., 1994; 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.

In our previous experiments, the CD147 mAbs used here demonstrated two different biological effects: induction of cell aggregation (Kasinrerk et al., 2000; Khunkeawla et al., 2001) and inhibition of cell proliferation (unpublished observations). M6-1D4, M6-1F3 and M6-2F9 mAbs induced U937 homotypic aggregation whereas M6-1B9 and M6-1E9 mAbs inhibited CD3 induced T cell proliferation (unpublished observations). These data implied that the epitopes recognized by the mAbs are bioactive domains which may interact with their native ligands. Western immunoblotting of CD147-ϕ indicated that the epitope recognized by M6-1B9, M6-1D4, M6-1E9 and M6-1F3 is a non-tertiary structure. However, epitopes for M6-2F9 and M6-2B1 mAbs are conformational. These results were in agreement with those observed using hemopoietic cell lysate (data not shown).

From the results of western immunoblotting (Fig. 3), competitive inhibition ELISA (Table 1) and our previous data of homotypic cell aggregation induction (Kasinrerk et al., 1999; Khunkeawla et al., 2001) and inhibition of T cell activation (unpublished observations), topographic information of CD147 bioactive epitopes on the CD147-ϕ was predicted (Fig. 4). MAbs M6-1B9 and M6-1E9 gave similar results for western immunoblotting and inhibition of T cell proliferation induced by CD3

mAb (unpublished observations), in as much as, competed with the same epitope area. Therefore, we proposed that the epitopes recognized by these mAbs are contiguous. On the contrary, the epitope of mAb M6-2F9 does not overlap or associate with the epitopes recognized by mAbs M6-1B9 and M6-1E9. This antigenic determinant is a bioactive domain for the induction of homotypic cell aggregation whereas the epitopes recognized by mAbs M6-1B9 and M6-1E9 conveyed the proliferation inhibition signal in T cell activation.

Surprisingly, mAb M6-1F3 was directed against a non-tertiary structure but stimulated cell aggregation as well. We presumed that the recognition sites of mAb M6-1F3 and M6-2F9 are adjacent, since the occupation of mAb M6-2F9 obstructed the binding of mAb M6-1F3. MAb M6-1F3 did not influence the binding of any mAbs tested, whilst mAbs M6-1B9, M6-1E9 and M6-2F9 interfered the capturing of mAb M6-1F3. We hypothesize based on the conformational change of antigen following mAb and epitope interaction (Davies and Cohen, 1996; Towbin et al., 1996) that the antigenic determinant of mAb M6-1F3 is comparably more rigid. Consequently mAb M6-1F3, as a competitor, was stabilized once bound to CD147 without swaying to hamper the recognition site of captured mAbs. In contrast, other mAbs introduced conformational changes, therefore masking the epitope area of mAb M6-1F3. To define the relationship of these epitopes, the epitope of mAb M6-1F3 was placed in the center surrounded by the other ones. However, another possibility which could not be excluded was the affinity difference of the CD147 mAbs. The binding affinity of mAb M6-1F3 maybe the weakest comparing to other tested CD147 mAbs, thus it could not interfere 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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Figure Legends

Figure 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.

Figure 2. Comparing the binding efficiency of CD147-ϕ derived from *E. coli* XL-1 Blue with 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 traced 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.

Figure 3. 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 arrow.

Figure 4. Topographic illustration of the predicted CD147 bioactive epitopes. Each geometric form represented individual epitope recognized by mAb. The intersections of the polygons were referred as the overlapping regions of the different epitopes.

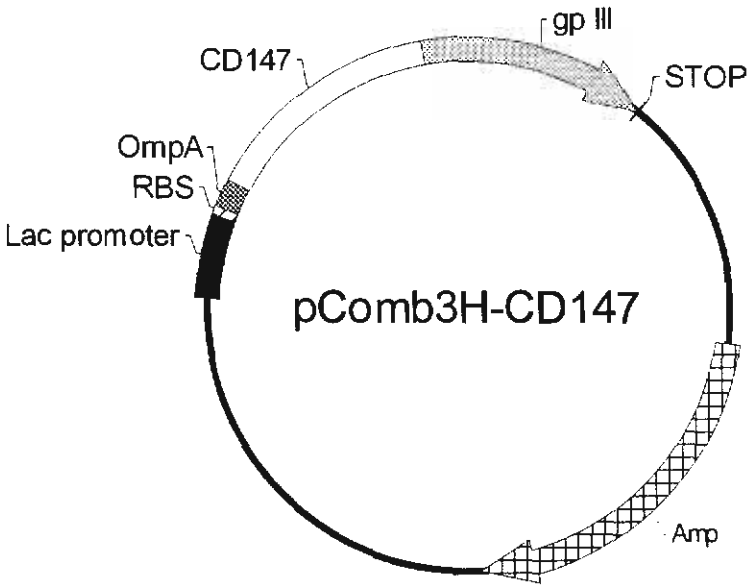

Figure 1

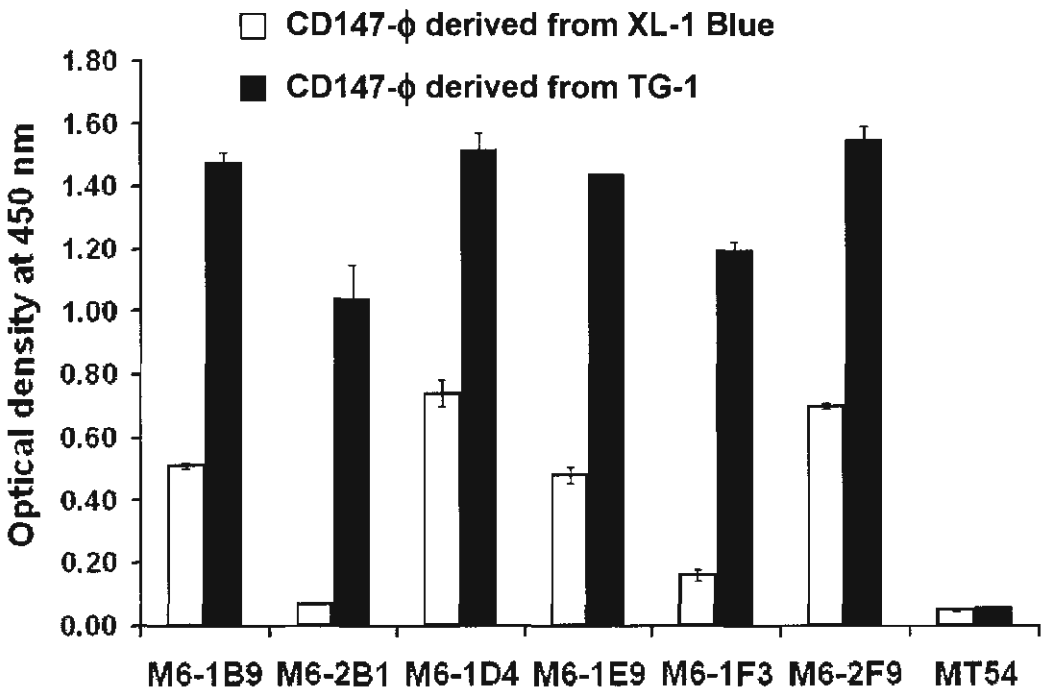
CD147-ϕ derived from XL-1 Blue
CD147-ϕ derived from TG-1
Optical density at 450 nm
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
M6-1B9
M6-2B1
M6-1D4
M6-1E9
M6-1F3
M6-2F9
MT54

Figure 2

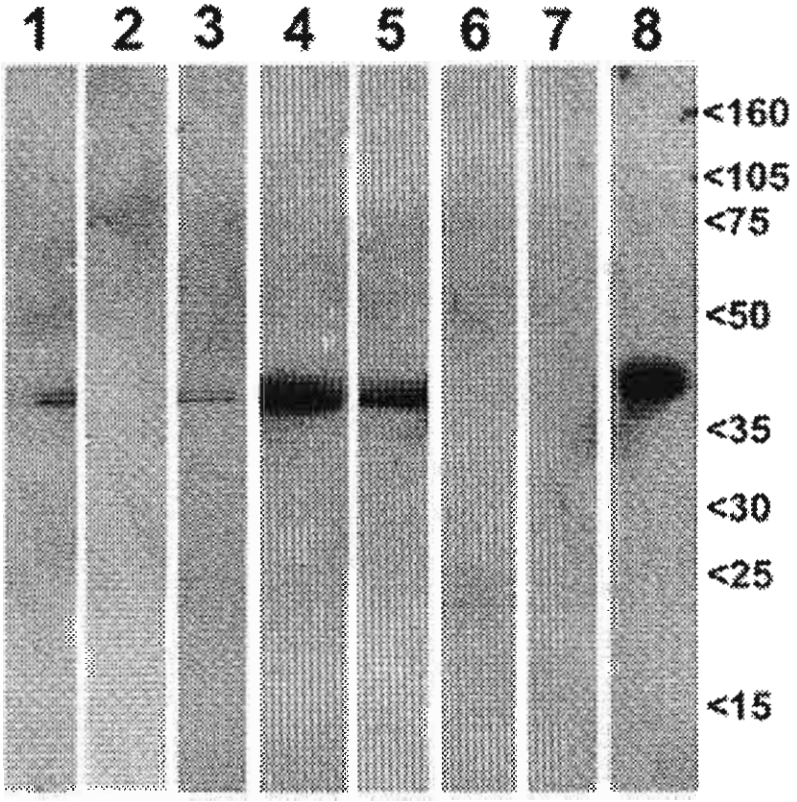

Figure 3

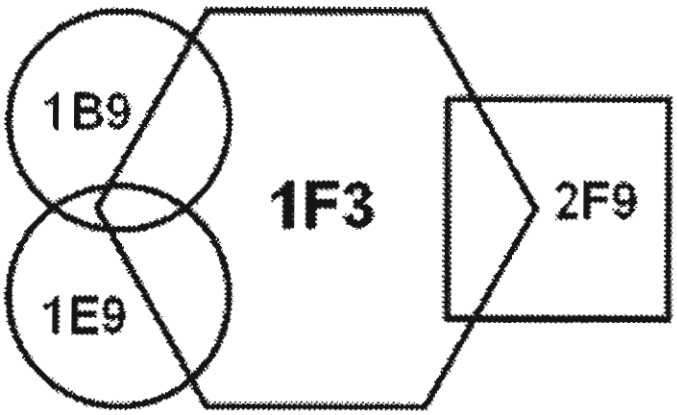

Figure 4

Table I 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.

Construction of high density display of CD147 ectodomain on VCSM13 phage via gpVIII: effects of temperature, IPTG and helper phage infection-period

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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; *Pel*B, pectate lyase B signal peptide from *Erwinia carotovora*

Abstract

Production of VCSM13 phage displaying high density of CD147 ectodomain (CD147Ex) was achieved when culturing conditions were modulated. Phagemid expressing CD147Ex was constructed and used to produce phage display CD147Ex gpVIII fusion protein in TG1 *E. coli*. Displaying of CD147Ex via gpVIII was successfully increased when growing the transformed TG1 at 25 °C with IPTG-stimulation. In addition to temperature and IPTG-stimulation, the VCSM13 helper phage infection-period was particularly affected the insertion of CD147Ex into phage progeny. By sandwich ELISA, incorporation of the CD147Ex into phage particle was confirmed. The correct size of the CD147Ex-gpVIII fusion protein at 28 kDa was demonstrated by western immunoblotting. Multivalent display of CD147Ex on phage particles will be valuable in further discovering of its ligand-partner.

Introduction

Phage display is a powerful technique for engineering proteins or peptides [1-5]. Proteins or peptides of interest are expressed as fusion to phage minor (gpIII) or major (gpVIII) coat proteins. In an order to identify CD147 counter-receptor, we recently applied phage display technology to construct phage-displayed CD147 on gpIII [6]. However, by using the constructed recombinant phages, we could not demonstrate the binding of phage-displayed CD147 to any cell types by immunofluorescence technique (unpublished observation). The weak binding affinity between CD147 and its ligand-partner was supposed. Increasing the copy number of CD147 molecule per phage particle may enhance the bonding forces by multivalent ligand-receptor anchoring and serve the purpose of ligand–receptor study.

Displaying of the heterologous proteins on phage gpIII limited the level of display to less than one molecule per phage particle [7]. In contrast, high level of heterologous protein display could be achieved on the gpVIII, which contains approximately 2,000 copies per phage particle [8-9]. The filamentous phage usually displayed the fusion protein at lower level than expected as a result of proteolysis of polypeptide was reported [10-13]. In general, phage is propagated at the optimal temperature for bacterial growth, 37 °C. This temperature, concurrently, is suitable for the enzymatic activity of proteases produced by *E. coli* host. The produced fusion proteins could be, then, degraded by these proteases and resulted in low amount of fusion proteins for packaging on phage particles. One strategy to prevent the degradation of the heterologous protein was to culture *E. coli* at low temperature i.e. 25 °C [14-16]. Lowering the temperature of phage propagation was formerly applied to overcome this problem [14, 17]. However, the rate of protein production was also significantly decreased at low temperature. To compensate this,

isopropyl thio-β-D-galactopyranoside (IPTG) was commonly added to up-regulate the expression level when a leaky inducible promoter such as *Lac* is provided [14, 18-21].

In an attempt to generated high density of phage expressing CD147, pComb8 phagemid vector was employed to display the ectodomain of human CD147 (CD147Ex) on gpVIII in the present study. The optimization of culturing conditions, i.e., temperature and IPTG for incorporating the CD147ExgpVIII into VCSM13 particle, was investigated. Since the packaging process is in the competitive manner between wild-type gpVIII and CD147ExgpVIII, we demonstrated also the effect of helper phage infection-period following the IPTG induction. The influences of these combinatorial factors on displaying of the heterologous protein on phage particle via gpVIII have never been reported elsewhere.

Materials and Methods

CD147Ex gene amplification by PCR

Two oligonucleotides, CD147ExgpVIIIFw (5′-GAG GAG GAG GTc tcg agG CTG CCG GCA CAG TCT TC-3′, the *Xho*I restriction site at 5′ overhang is designated in small letters) and CD147ExgpVIIIRev (5′-GAG GAG GAG CTa cta gtG TGG CTG CGC ACG CGG AG-3′, the *Spe*I restriction site at 5′ overhang is designated in small letters), were used as primers for amplifying the CD147Ex fragment.

PCR was used to amplify the CD147Ex from the mammalian expressing vector, pCDM8-CD147 [22]. Fifty nanograms of pCDM8-CD147 was annealed with 250 ng of each described primer in the 100 μl of a PCR mixture containing 5 U of ProofStart DNA polymerase (QIAGEN, Hilden, Germany). The PCR cycling condition was one cycle at 95 °C for 5 min followed by 34 cycles of 94 °C for 50 sec, 50 °C for 50 sec, and 72 °C for 1 min. After 35 amplification cycles, the mixture was incubated at 72 °C for 10 min. The resulting 550 bp PCR product was subsequently treated with *Xho*I and *Spe*I at 37 °C for 18 h and purified by QIAquick PCR purification Kit (QIAGEN, Hilden, Germany).

Construction of phagemid expressing CD147Ex

The pComb8 phagemid (a gift from Dr. C.F. Barbas, Scripps Institute, USA) was digested with *Xho*I and *Spe*I at 37 °C for 18 h. The DNA fragment of digested pComb8, 3300 bp, was further purified and ligated with digested CD147Ex PCR product by using T_4 ligase enzyme (Roche Molecular Biochemicals, Manheim, Germany). The ligated product was transformed into TG1 *E. coli* host strain {*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F' [*traD36proAB+*, *lacI*^{q} *lacZ*ΔM15]} (kindly provided by Dr. A.D. Griffiths, MRC

Cambridge, UK). The transformed *E. coli* were selected on LB agar containing ampicillin (100 μg/ml). The ampicillin resistant colonies were selected and cultured at 37 °C for 18 h in LB broth containing ampicillin (100 μg/ml) for Plasmid Mini Kit (QIAGEN, Hilden, Germany). The inserted CD147Ex gene in the purified phagemid was checked by restriction fragment analysis with *Xho*I and *Spe*I, and reamplification by PCR. The newly constructed phagemid was named pComb8-CD147Ex.

Phage-displayed CD147ExgpVIII

Transformed bacteria were grown in 10 ml of 2xTY broth (1.6%[wt/vol] tryptone, 1%[wt/vol] yeast extract, and 0.5%[wt/vol] sodium chloride) containing ampicillin (100 μ g/ml) at 37 °C until the absorption at 600 nm was 0.8. The precultured bacteria were subsequently transferred to 100 ml of the same medium containing 1%[wt/vol] glucose and cultivated until the absorption at 600 nm of 0.5 was reached at either 25 or 37 °C. If the bacterial cultures were to be induced, IPTG was added to the culture at a final concentration of 1 mM. After induction, the cells were grown at the desired temperature for 0 or 2 h. Each bacterial culture was further infected with 10^{12} t.u. of the VCSM13 helper phages and left at 37 °C for 30 min without shaking. Phage-infected TG1 were pelleted by centrifugation at 1,100 g for 10 min at 4 °C. The pellets were resuspended in 2xTY broth containing ampicillin (100 μg/ml) and kanamycin (70 μg/ml). Fifteen milliliters of bacterial cultures were transferred to 250 ml of the same medium and shaken at 180 rpm for 16 h at the desired temperature. The recombinant phages were harvested by PEG 8000 precipitation and centrifugation as described previously [23]. Finally, the phage were reconstituted with 2.5 ml of 0.15 M PBS pH 7.2 and centrifuged at 11,600 rpm for 10

min at 4 °C. The supernatant was stored at -70 °C. Overall experiments were simplified by the diagram (Fig. 1).

Immunoassay for phage-displayed CD147ExgpVIII by sandwich ELISA

Fifty microliters of 10 μg/ml CD147 mAbs (M6-1B9; IgG_3, M6-1E9; IgG_{2a}, M6-1D4; IgM, and M6-2F9; IgM) [24-25] in carbonate/bicarbonate buffer pH 9.6 were coated on microtiter plate (NUNC, Roskilde, Denmark) at 4 °C for 18 h. Blocking was performed by addition of 2% skimmed milk in PBS pH 7.2 and incubated for 1 h at room temperature. After washing 5 times with 0.05% Tween20 in PBS pH 7.2, phage-displayed CD147ExgpVIII produced under different conditions were diluted to the optimal concentration in 2% skimmed milk in PBS pH 7.2 and added to the wells. After incubation at room temperature for 1 h, the plate was washed to remove unbound phages. Binding of phage particles was monitored by using two alternative sets of anti-M13 mAb. One was HRP-conjugated anti-gpVIII mAb (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the other was biotinylated anti-gpIII mAb (Exalpha Biologicals, Watertown, MA). HRP-conjugated Streptavidin (ZYMED Laboratories, San Francisco, CA) was applied to trace the biotinylated anti-gpIII mAb. Following washing, the 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for signal development. Optical density at 450 nm was determined after adding 1 N HCl to stop the reaction.

SDS-PAGE and Western Immunoblotting

Phage-displayed CD147ExgpVIII was separated by SDS-PAGE under reducing conditions on a 15% acrylamide gel. For western immunoblotting, the separated proteins were electroblotted onto polyvinylidene-fluoride (PVDF) membrane. Blotted membrane was blocked at 4 °C for 18 h in 5% skimmed milk in PBS pH 7.2 then incubation with the pooled CD147 mAbs (M6-1B9, M6-1E9, and M6-1D4 at 10 μg/ml each) for 1 h at room temperature on a shaking platform. Following washing with 0.05% Tween20 in PBS pH 7.2, HRP-conjugated rabbit-anti-mouse immunoglobulins (DAKO A/S, Copenhagen, Denmark) was added to the membranes and incubated at room temperature for 1 h. The immunoreactive bands were then visualized by chemiluminescent detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Results

Construction of pComb8-CD147Ex phagemid

The ectodomain of CD147 cDNA was amplified from pCDM8-CD147 by PCR using primers CD147ExgpVIIIFw and CD147ExgpVIIIRev. The PCR product of 552 bp was obtained (Fig. 2). The amplified product was subsequently cleaved with *Xho*I and *Spe*I, and ligated into the pComb8 phagemid treated with the same enzymes. The ampicillin-resistant *E. coli* TG1 colonies transformed with the ligated product were grown at 37 °C for plasmid minipreps. The inserted fragment of CD147Ex with the molecular weight of approximately 550 bp was retrieved from the isolated plasmid DNA after checking by restriction fragment analysis with *Xho*I and *Spe*I (Fig. 3), and reamplification by PCR (Fig. 4). The engineered phagemid bearing CD147 ectodomain gene, flanked upstream by *Pel*B signal sequence and downstream by gpVIII (Fig. 5), was designated as pComb8-CD147Ex.

Detection of phage-displayed CD147ExgpVIII by sandwich ELISA

To study the culturing conditions for efficiently displaying of CD147Ex molecules on phage particle, the pComb8-CD147Ex-transformed TG1 was cultured and infected with the VCSM13 helper phages under five different conditions (Fig. 1). The phage-displayed CD147ExgpVIII in culture supernatants obtained from various culturing conditions were compared by sandwich ELISA using a panel of anti-CD147 mAb as capture antibody. By using HRP-conjugated anti-gpVIII mAb, production of phage-displayed CD147ExgpVIII at 25 °C with IPTG-stimulation showed the highest signal with all anti-CD147 mAbs (M6-1B9, M6-1E9, M6-1D4, and M6-2F9) used (Fig. 6). The second order was phage harvested from 37 °C without IPTG-, followed by 25 °C without IPTG-, and 37 °C with IPTG-

stimulation when mAb M6-1B9 was used as a capture antibody. However, the second and the third rank were switched when the precipitated phages were captured by other three mAbs (M6-1E9, M6-1D4, and M6-2F9). In contrast, the recombinant phages were not recognized by an irrelevant capture antibody, mouse anti-GFP mAb.

The binding of the phage-displayed CD147ExgpVIII under 25 °C with IPTG-stimulation onto the CD147 mAb-coated wells was also demonstrated by biotinylated anti-gpIII mAb/HRP-conjugated Streptavidin detection system (Fig. 7). The signaling pattern of all capture-mAbs used was corresponded to the HRP-conjugated anti-gpVIII mAb detection system.

To determine the effect of helper phage infection-period, the phage-displayed CD147ExgpVIII were produced under 25 °C with IPTG-stimulation for 0 h and 2 h before helper phage infection. The produced phages from both conditions were compared by sandwich ELISA. The recombinant phages harvested from simultaneous IPTG-stimulation and helper phage infection (0 h) was 6-11-fold higher than those harvested from which inoculation of helper phage 2 h after IPTG-stimulation (Table 1).

Western Immunoblotting

The protein components of the recombinant phages prepared under 25 °C with IPTG-stimulation was fractionated by SDS-PAGE under reducing conditions, blotted and probed with pooled CD147 mAbs (M6-1B9, M6-1E9, and M6-1D4). An immunoreactive protein band with the molecular weight of 28 kDa was obtained (Fig. 8). This appeared band was regarded as the fusion protein of CD147Ex, 20 kDa and gpVIII, 6 kDa. No reactive band was detected in the control lane applied with phage-displayed Fo protein of respiratory syncytial virus.

Discussions

In an attempt to characterize the counter-receptor of CD147 molecule, we recently generated phage-displayed ectodomain of CD147 via gpIII [6]. However, by indirect immunofluorescence, the binding of these recombinant phages on any cells could not be demonstrated. Since the majority of the generated phage possessed less than one copy of CD147 molecule, the low ligand-binding affinity of the CD147 to its ligands was presumed. Multimeric display of CD147-on phage particles are therefore required and will be valuable in further discovering its ligand-partner on cell surface. Furthermore, multivalent CD147 on the 930 nm-length filamentous phage might overwhelm the limitation in the binding of CD147 to the low-level expression of its ligands. The multivalent phage carrying CD147 may be valuable in functional study. Signal transferring by individual pairing with monovalent CD147 may differ from the cross-linking of cell-surface ligands with phage-displayed multivalent CD147 via gpVIII. CD147 appears to be a cofactor that mediates activity of virus-associated cyclophilin A and is required for efficient infection by HIV-1 have recently be demonstrated [7]. Phage-displayed CD147 might be used to define the novel targets for anti-HIV interventions.

The gpVIII of VCSM13 was previously applied for achieving the multimeric polypeptide libraries [8-9]. The absolute binding force of these phage libraries to a certain ligand was enhanced according to the increasing copy number of each polypeptide. The displaying of CD147 ectodomain on phage particles via gpVIII was, thus, proposed in this study. However, the multivalent display of fusion protein on phage particles had been hindered because of a limitation on the fusion length [26]. Culturing conditions during phage preparation such as temperature, IPTG-stimulation and helper phage infection-period are important factors for production of the phage expressing interested protein. In

the present study, the factors mentioned were investigated to optimize the quantity of display. Filamentous phage was used to display CD147EXgpVIII in TG1 *E. coli* host. By sandwich ELISA using a panel of anti-CD147 monoclonal antibodies as a capture and HRP-conjugated anti-gpVIII mAb as a tracer, the generated phages showed strong positive reactivity. The correct size of the CD147Ex-gpVIII fusion protein at 28 kDa from the generated phages was also demonstrated by western immunoblotting. This result indicated that the expression of fusion CD147-gpVIII proteins was occurred. However, we raised a question whether the positive signal was due to the secreted or the phage-bound CD147Ex-gpVIII fusion protein. To address this question, the expression of CD147 on the surface of complete phage particles was subsequently confirmed by using the biotinylated anti-gpIII mAb/HRP-conjugated Streptavidin as a tracer in sandwich ELISA. As predicted, the generated phages showed also strong positive reactivity. Our ELISA experiments indicated that the generated phages harbored CD147 ectodomain on their surfaces.

Production of phage-displayed CD147ExgpVIII at 25 °C with IPTG-stimulation was demonstrated the most suitable condition. Reducing growth temperature, from 37 °C to 25 °C, may facilitate the correct folding and stability of the displayed protein [27-29]. Although the rate of protein synthesis was low at 25 °C comparing to 37 °C, IPTG seemed to compensate this disadvantage. The reduction in cultivating temperature and addition of IPTG may have a complementary effect on phage propagation and on the fusion protein incorporation resulting in the high density display per phage particle.

Phage harvested from 37 °C without IPTG-stimulation was demonstrated to be the second effective order when M6-1B9 mAb was used as a capture antibody. In surprising contrast, when other three mAbs (M6-1E9, M6-1D4, and M6-2F9) were used as capture antibodies, the second rank was changed to phage harvested from 25 °C without IPTG-

stimulation. We speculated that the folding of CD147 epitope recognized by M6-1B9 mAb on recombinant phage produced at 37 °C without IPTG-stimulation was more proper than phages produced at 25 °C without IPTG-stimulation. Regarding our previous study [6], the epitopes recognized by M6-1B9 and M6-1E9 mAbs were overlapped. The variation in exposing of these epitopes in the different culturing conditions indicated the proximate but non-identical epitopes recognized by M6-1B9 and M6-1E9 mAbs. The recombinant phage harvested from 37 °C with IPTG-stimulation was given the lowest signal in all capture CD147 mAbs used. This result was consistent with the experiment of antibody-gpVIII phage production [19]. Insoluble protein accumulation and degradation of the recombinant protein in the periplasm by host proteases might be facilitated at higher growth temperatures [28]. Apparently, high expression level of fusion protein by IPTG-stimulation rendered the formation of inclusion bodies which decreased the incorporating efficiency of CD147Ex-gpVIII into phage particles [27-29].

One proposed strategy for increasing the amount of recombinant protein displayed on phage particles via gpVIII was by delaying the infection-period of helper phage after IPTG-stimulation [18]. However, the controversial result was obtained in this study. The phage-displayed CD147ExgpVIII produced at 25 °C with IPTG-stimulation at the same time of helper phage infection were higher than those obtained by infection of helper phages 2 h later. We suggested that after 2-h induction, *E. coli* produced an excess amount of CD147ExgpVIII which was prone to form aggregation before the phage packaging components were synthesized.

In order to produce high density phages, various sophisticate techniques have been developed. Sidhu increased the display of human growth hormone by mutating the amino acid residues on the gpVIII fusion partner [30]. More recently, a novel gpVIII display

system, in which every copy of gpVIII was modified, was reported [21]. The gpVIII was expressed from the phagemid and the helper phage used to rescue the phagemid had been deficient in gpVIII production. In contrast, we established here a simple method for increasing of the multimeric CD147Ex expression on phage particles. Apart from the major parameters i.e. temperature and IPTG-stimulation which were formerly investigated [14, 19], the influence of helper phage infection-period after IPTG-stimulation on enhancing the CD147Ex displaying, particularly, demonstrated a high impact of this study.

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Figure Legends

Figure 1. Schematic diagram of the cultivating conditions for production of phage-displayed CD147ExgpVIII.

Figure 2. Analysis of PCR product, 552 bp (*), of the CD147Ex amplified from pCDM8-CD147 vector using CD147ExgpVIIIFw and CD147ExgpVIIIRev primers. Samples were electrophoresed in a 1% agarose gel. Lane 1, 1 kb DNA marker; lane 2, amplified product.

Figure 3. Restriction fragment analysis of pComb8-CD147Ex with *Xho*I and *Spe*I. The constructed pComb8-CD147Ex was cleaved with *Xho*I and *Spe*I. The inserted fragment of CD147Ex with the molecular weight of approximately 552 bp (*) was retrieved from the pComb8-CD147Ex (lane 3). Lane 1, 1 kb DNA marker; lane 2, undigested pComb8-CD147Ex; lane 3, *Xho*I- and *Spe*I-digested pComb8-CD147Ex.

Figure 4. Amplified product of CD147Ex from pComb8-CD147Ex. The 552 bp amplified product (*) from pComb8-CD147Ex template using CD147ExgpVIIIFw and CD147ExgpVIIIRev primers is shown (lane 2). Lane 1, 1 kb DNA marker; Lane 2, amplified product.

Figure 5. Construction of pComb8-CD147Ex phagemid. *Xho*I- and *Spe*I-cloning sites where the CD147Ex gene was inserted; *lac* promoter, the signal sequence (*Pel*B) and gpVIII gene are shown. Ampicillin is used as a selection marker.

Figure 6. Sandwich ELISA analysis of phage-displayed CD147ExgpVIII under different growth conditions using four anti-CD147 mAbs (M6-1B9, M6-1E9, M6-1D4, and M6-2F9) as a capture and HRP-conjugated anti-gpVIII mAb as a tracer. Phage-displayed CD147Ex under 37 °C with IPTG-stimulation (no fill), 37 °C (solid bars), 25 °C with IPTG-stimulation (horizontals) and 25 °C (diagonals) were demonstrated. This experiment was done in duplicate.

Figure 7. Sandwich ELISA analysis of phage-displayed CD147ExgpVIII under 25 °C with IPTG-stimulation using four anti-CD147 mAbs (M6-1B9, M6-1E9, M6-1D4, and M6-2F9) as a capture and biotinylated anti-gpIII mAb/HRP-conjugated Streptavidin as a tracer.

Figure 8. Western immunoblotting of phage-displayed CD147ExgpVIII separated by reducing SDS-PAGE. Immunological assay was performed by probing with pooled anti-CD147 mAbs (M6-1B9, M6-1E9, and M6-1D4). Lane 1, phage-displayed CD147ExgpVIII; lane 2, phage-displayed Fo protein of respiratory syncytial virus. The immunoreactive bands were visualized by chemiluminescent detection system. Molecular weight markers in kDa were indicated.

Figures

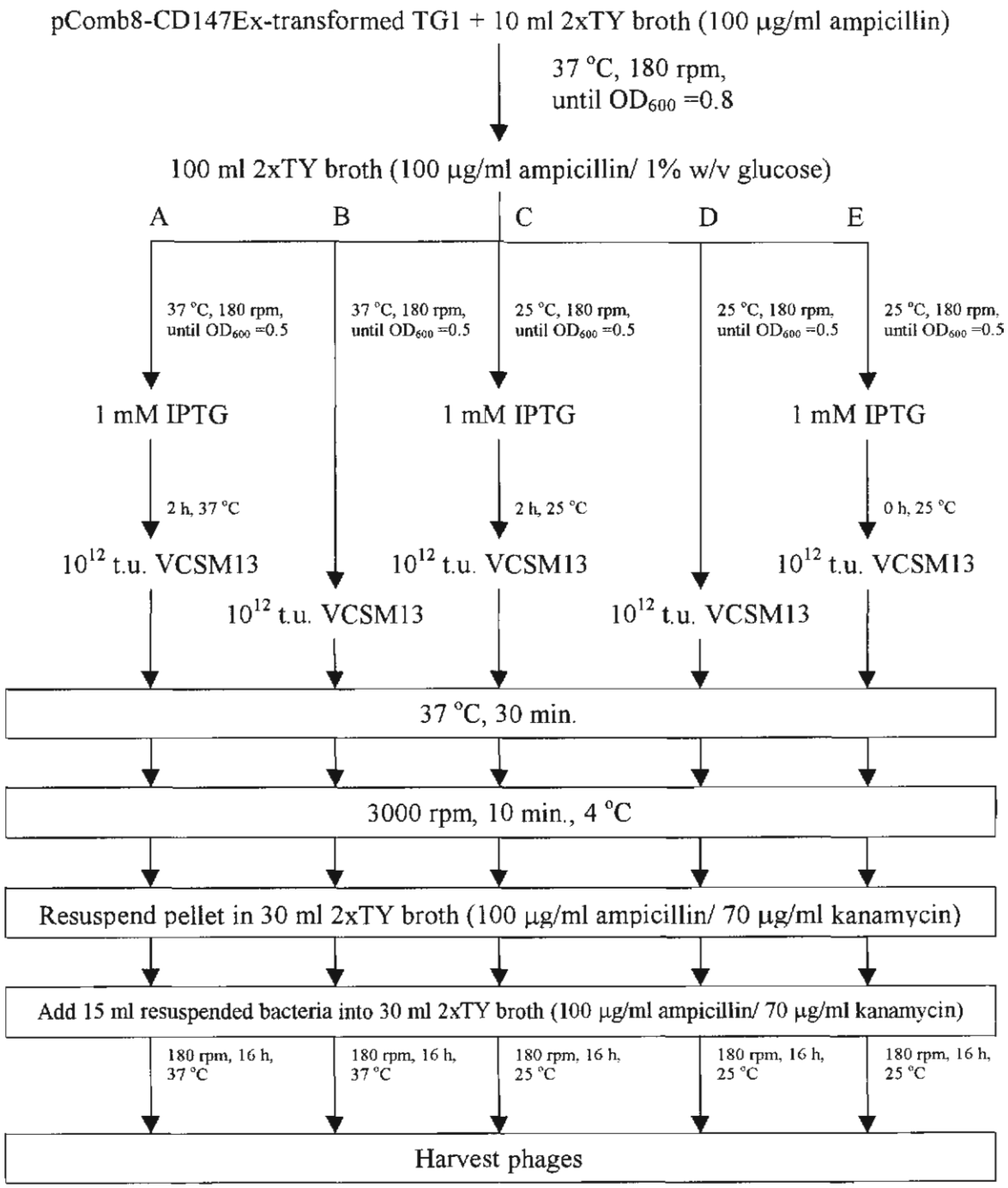


Fig. 1

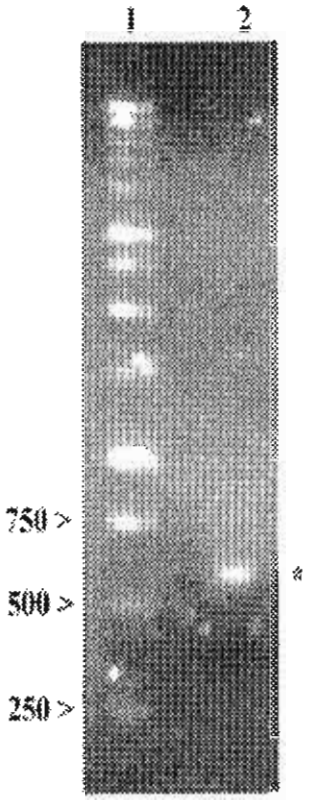


Fig. 2

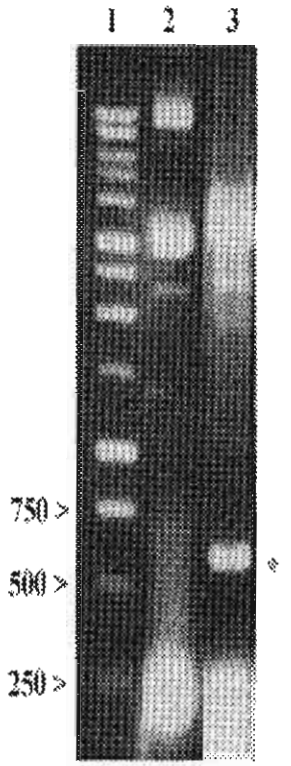


Fig. 3

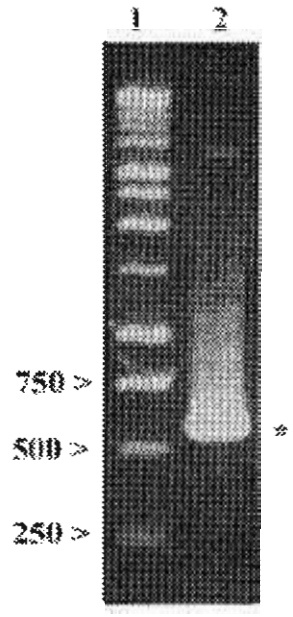


Fig. 4

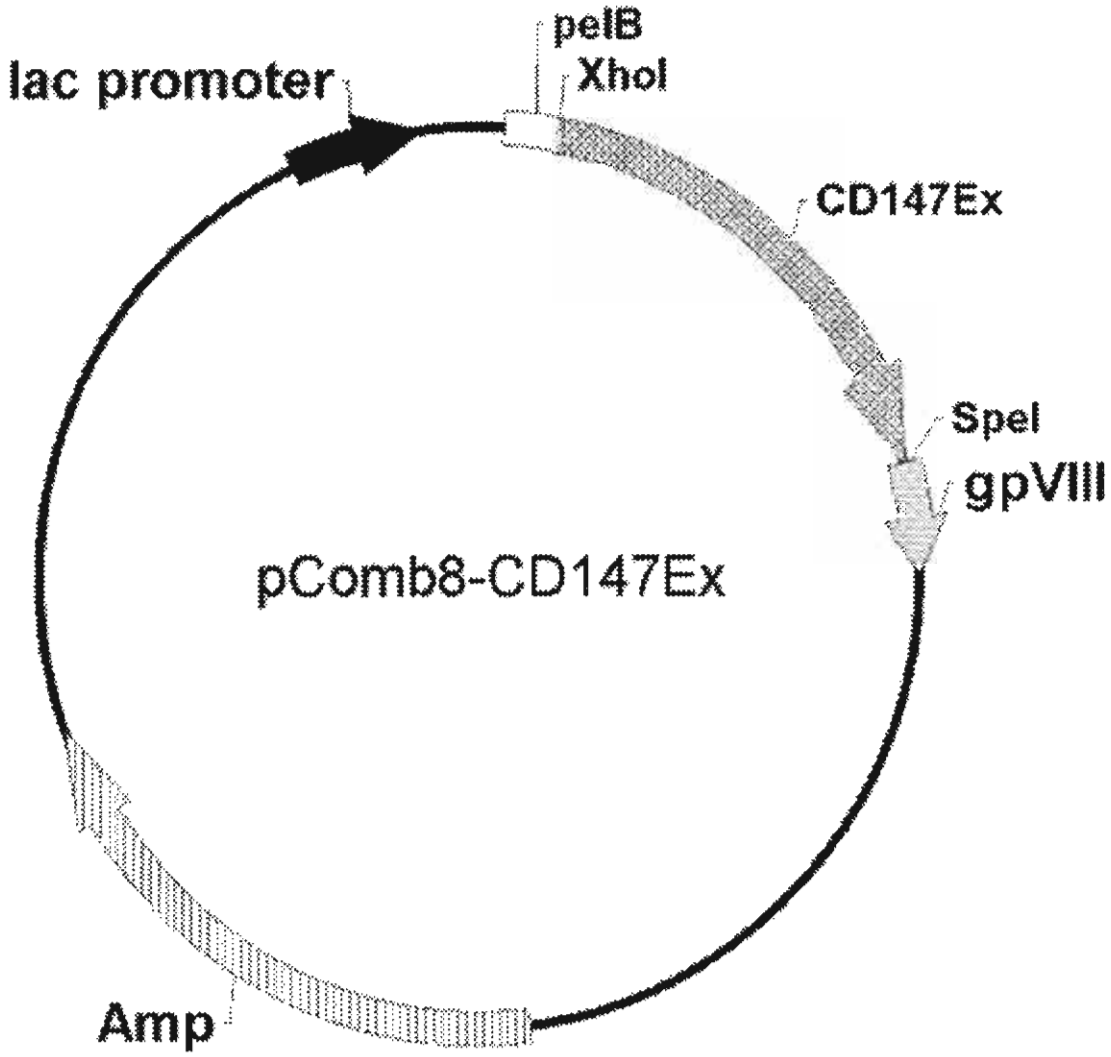

lac promoter
pelB
XhoI
CD147Ex
SpeI
gpVIII
pComb8-CD147Ex
Amp

Fig. 5

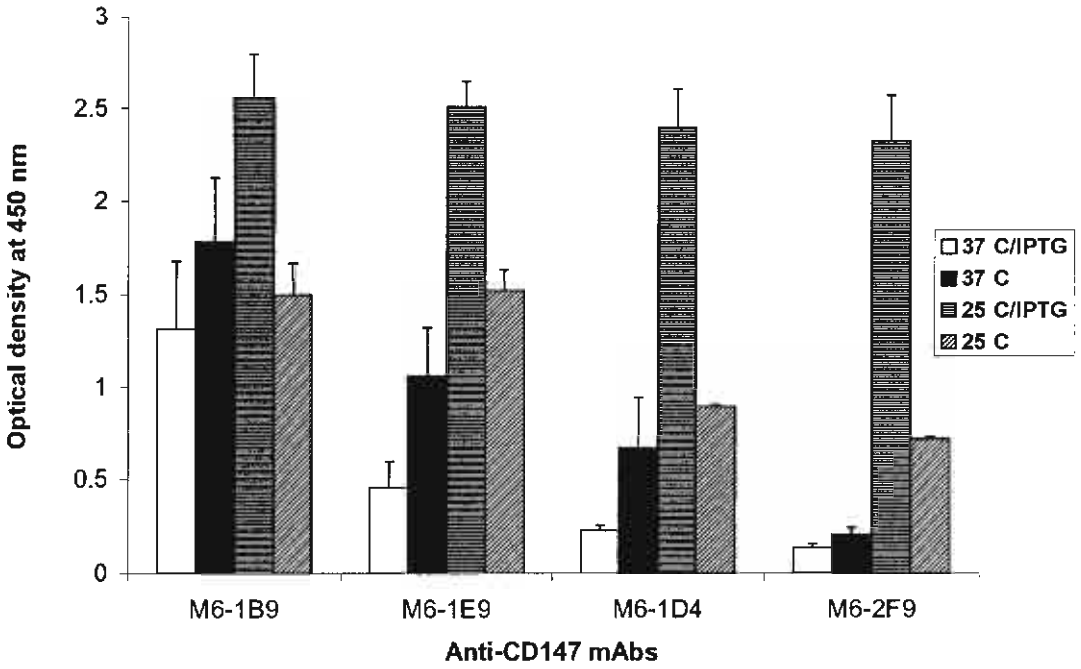
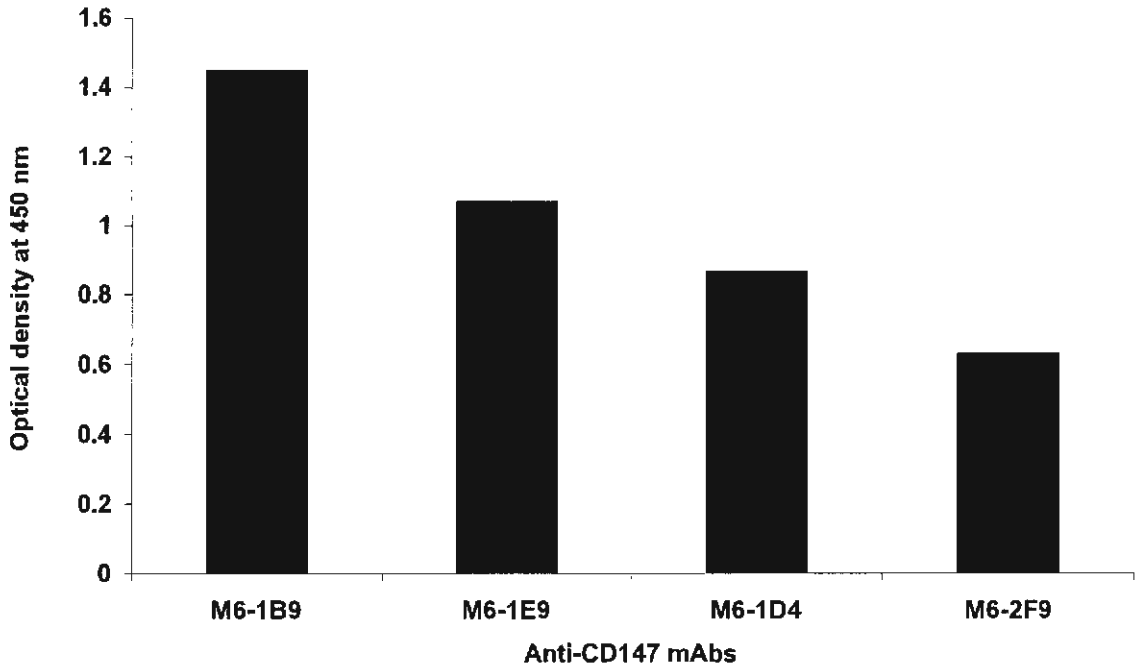

Optical density at 450 nm
3
2.5
2
1.5
1
0.5
0
M6-1B9
M6-1E9
M6-1D4
M6-2F9
Anti-CD147 mAbs
37 C/IPTG
37 C
25 C/IPTG
25 C

Fig. 6


Optical density at 450 nm
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1.4
1.2
1
0.8
0.6
0.4
0.2
0
M6-1B9
M6-1E9
M6-1D4
M6-2F9
Anti-CD147 mAbs

Fig. 7

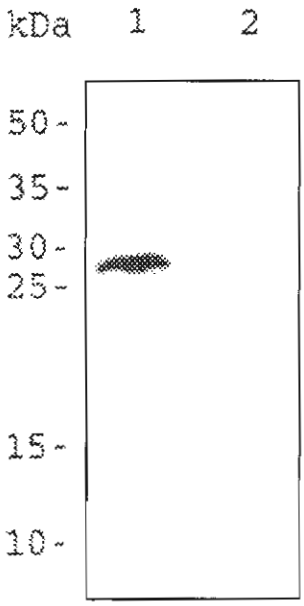

Fig. 8

Table 1. Comparison of helper phage infection-periods on the production of phage-displayed CD147ExgpVIII at 25 °C with IPTG-stimulation.

CD147 mAbs	Optical density at 450 nm	
	0 h after IPTG-stimulation	2 h after IPTG-stimulation
M6-1B9	1.68	0.28
M6-1E9	1.47	0.13
M6-1D4	1.67	0.19
M6-2F9	1.24	0.20

Sandwich ELISA using a panel of CD147 mAbs as a capture and tracing by biotinylated anti-gpIII mAb/HRP-conjugated Streptavidin system was employed.

P50

PRODUCTION AND EPITOPE CHARACTERIZATION OF PHAGE-DISPLAYED LEUKOCYTE SURFACE MOLECULE: CD147

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CD147 is broadly expressed cell surface glycoprotein of Ig supergene family whose expression is upregulated upon T cell activation. Its immunological roles are still unclear. The phage-displayed CD147 was produced by cloning the CD147 cDNA coding for the external domain into the site between OmpA and gpIII of phagemid, pComb3HSS. The constructed vector, pComb3H-CD147, was separately transformed into either *E. coli* XL-1 Blue or TG-1 strain. The transformed hosts were cultured and infected with VCSM13. The recombinant phages were harvested and evaluated by immunological techniques. According to sandwich ELISA, more effective folding of the CD147 on recombinant phage produced from TG-1 was clearly demonstrated. By native Western immunoblotting, 5 of 6 mAbs directed against CD147 bound to the disulfide-bond dependent epitopes of CD147-gpIII fusion molecule localizing at 43 kDa. However, none of them reacted with linear peptide epitope. The epitope mapping was performed by using 6 mAbs in competitive inhibition study with phage-displayed CD147 molecule. Consequently, by bioneutralizing assay, the different binding sites were indicated and the epitope map was created. This study demonstrated the application of phage display technique for epitope mapping. In addition, the awareness of using the suitable host for displaying the recombinant molecules was also depicted.

P51

ENGAGEMENT OF CD147 MOLECULE BY SPECIFIC MONOCLONAL ANTIBODIES SUPPRESS T LYMPHOCYTE ACTIVATION

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CD147 is a leukocyte surface molecule which is belong to the immunoglobulin superfamily. It is broadly expressed on various cell types and is a lymphocyte activation associated molecule. In order to study the function of CD147 molecule, in this study, 6 monoclonal antibodies (mAbs) against CD147 molecules were generated. By western blotting and cross blocking experiments indicated that these CD147 mAbs react to different epitopes on the CD147 molecule. All of these CD147 mAbs did not induced apoptosis of U937, Sup T1 and KG1 cell lines. However, engagement of CD147 molecule by 2 out of 6 generated CD147 mAbs down-regulated the *in vitro* T lymphocyte proliferation induced by CD3-specific OKT3 mAb. These results indicated that CD147 molecule is involved in regulation of T lymphocyte proliferation. Specific epitope on the CD147 molecule is required in the controlling T lymphocyte activation.