CHAPTER IV

DISCUSSION

High affinity binding interaction of biotin to avidin/streptavidin has been used widely in biochemistry and molecular biology, often in sensitive protein detection or protein capture applications. However, *in vitro* chemical techniques for protein biotinylation are not always successful, with some common problems being a lack of reaction specificity, inactivation of amino acid residues critical for protein functions and low level of biotin incorporation. Herein, we described the intracellular expression and concomitant site-specific *in vivo* biotinylation of the extracellular portion of human CD147. This approach is based on the cytoplasmic expression of CD147Ex fused to the biotin acceptor domain of *E. coli* BCCP which is a natural substrate of biotin ligase that catalyse the conjugation of the biotin moiety to a specific lysine residue in the BCCP. A prerequisite for the success of this approach was the use of the redox-modified *E. coli* strain with oxidizing cytoplasm where the formation of the important disulfide bridges can occur. Recently, by using similar strategy, successful cytoplasmic expression of functional single chain Fv antibody fragment with adequate biotinylation has been described (Santala and Lamminmaki, 2004). The biotinylated antibody was demonstrated to be applicable in immunoassay experiments with the comparable sensitivity to the currently used commercial test kits (Santala and Lamminmaki, 2004).

In this study, the BCCP biotin acceptor domain was cloned in frame and downstream to the human CD147 ectodomain (CD147Ex) coding sequence. The CD147Ex-BCCP fusion protein was expressed in *E. coli* Origami B after culturing at 25^{o}C with IPTG induction. The biotinylation of recombinant molecule obtained in bacterial extract was verified using indirect ELISA. All of CD147 mAbs recognized the fusion protein captured on ELISA plate that coated with egg white avidin, indicating the presence of CD147Ex-BCCP with biotin attached. In contrast, there was non-significant signal on the well applied with crude extract from survivin-BCCP-expressing or mock transformed Origami B. This result excluded non-specific binding of CD147 mAbs to the irrelevant proteins. Furthermore, the binding of two CD147 mAbs (M6-2B1 and M6-2F9) which is known to react with conformational epitopes (Tayapiwatana et al., 2003) indicated the proper folding of CD147 in high oxidizing condition of Origami B cytoplasm. In previous CD147 functional study, Kasinrerk and colleagues reported the signaling effects of CD147 mAbs (M6-1D4, M6-1F3 and M6-2F9) in inducing homotypic cell aggregation of U937 (Kasinrerk et al., 1999). Since CD147Ex-BCCP was strongly recognized by all of the CD147 mAbs, those bioactive epitopes of CD147 were reserved in the fusion molecule. This suggests that CD147Ex-BCCP may be valuable in further studies of CD147 biological functions or other applications that the glycosylation of the protein is not required. The expected protein band (approximately 30 kDa) was also detected by CD147 mAbs and HRP-conjugated streptavidin in Western immunoblotting, confirming the presence of biotinylated CD147Ex-BCCP fusion protein. These results suggested the efficient cytoplasmic expression of CD147Ex-BCCP with adequate *in vivo* biotinylation. Since the protein was biotinylated outside the CD147Ex segment, the

overall conformation and native epitopes were preserved. In comparison with chemical biotinylation, the antigenicity of the target molecule may be altered upon conjugation.

It has been reported that cytoplasmic expression of recombinant proteins as misfolded or aggregated protein products could be observed even though in the redox-modified *E. coli* strains (Jurado et al., 2002; Xiong et al., 2005). Indeed, Bessette et al. have shown that the kinetics of protein oxidation in the cytoplasm of FA113, the *E. coli* strain that carry the similar genetic modifications of the disulfide-reducing pathways as our host Origami B, is still fairly slow compared to that in the periplasm (Bessette et al., 1999). Thus, an insufficient oxidation capacity to promote the formation of disulfide bonds may be one of the reasons for the misfold and aggregation of the overexpressed recombinant proteins. A possible way to improve the expression level of recombinant proteins in the cytoplasm has been demonstrated by Jurado et al. (Jurado et al., 2002). The cytoplasmic co-expression of the periplasmic chaperone disulfide isomerase (DsbC) along with a single chain Fv improved the yield of the functional antibody expression. In our case, efficient soluble expression of CD147Ex-BCCP was simply achieved by using low induction temperature (i.e. 25°C), primarily due to the slower protein production rate. Alternatively, the expression rate might be adjusted by optimizing the IPTG concentration, the strategy that could be useful in the further studies.

Regarding the use of biotin acceptor fusion tags, many works have experienced the insufficient capacity of the *E. coli* machinery to serve the overexpressed recombinant proteins biotinylated (Weiss et al., 1994; Tucker and Grisshammer, 1996). Because the co-expression of the BirA biotin ligase successfully

increased the biotinylation level of the fusion proteins containing its substrate segments, the observed incomplete biotinylation is likely resulted from the limited endogenous biotin ligase activity (Tucker and Grisshammer, 1996; Sibler et al., 1999). Recently, Santala and Lamminmaki found that, if adequate amount of supplementary biotin was provided (i.e. 4 μM; the concentration used in this study), the endogenous biotin ligase activity alone was high enough for efficient biotinylation and that the biotinylation level of the recombinant proteins was mainly dependent on the availability of biotin (Santala and Lamminmaki, 2004). In agreement with this finding, without any supplementary biotin, the partial biotinylation of the recombinant protein fused to 15 amino acids biotin acceptor peptides (AviTag; Avidity LLC) was also observed even though the biotin ligase was co-expressed (Scholle et al., 2004). While the biotinylation level of CD147Ex-BCCP was not directly measured, the complete biotinylation of the fusion molecule seems to be unnecessary to our strategy. The nearly covalent interaction of biotin with avidin or streptavidin could be used to capture only the biotinylated form of fusion protein to the solid phases (e.g. microtiter wells). As demonstrated by indirect ELISA, the CD147 mAbs strongly reacted with CD147Ex-BCCP in the egg white avidin-coated wells, but not for the uncoated wells. This observation indicated the immobilization of CD147Ex-BCCP via biotin-avidin interaction. This advantage could be usefully utilized, for example, for protein capture or immuno-detection applications.

Several fusion tags are generally employed for recombinant protein detection and purification purposes. The hexa-histidine (His_6) tag, one of the most commonly used affinity tags, has an advantage in small size and inexpensive purification matrix but the chemical reagent used to elute the bound proteins (i.e. imidazole) may

influence the subsequent experiments such as NMR studies, or often results in protein aggregation (Hefti et al., 2001). In case of maltose-binding protein (MBP), it promotes the solubility of recombinant proteins and can be purified by amyrose resin (Kapust and Waugh, 1999). However, the molecular size is very high (40 kDa), consuming more substrate for protein production. The glutathione S-transferase (GST) tag also has bulky size and tends to form dimers, which can complicate the purification of the fusion proteins (Kaplan et al., 1997). The biotin domain of BCCP, the fusion tag used here, has advantages in its medium size (i.e. 85 amino acids, 9 kDa) and the capability to be biotinylated *in vivo* by the endogenous machinery of *E. coli* at the specific site within the BCCP segment. The BCCP domain fusion approach has previously been used for site-specific biotin labeling of various recombinant proteins (Min et al., 1999; Sibler et al., 1999; Santala and Lamminmaki, 2004).

By screening peptide libraries for the substrates of biotinylation enzymes, Schatz generated a small 13 amino acids peptide that mimic the folded conformation of the BCCP biotin acceptor domain and which is itself the artificial substrate for the *E. coli* biotin ligase (Schatz, 1993). This peptide has been used for the enzymatic biotinylation of various recombinant proteins both as an N- and a C-terminal fusion (Tsao et al., 1996; Tucker and Grisshammer, 1996; Smith et al., 1998). However, the use of BCCP domain as fusion tag may advantage over the small peptide as the avidin/streptavidin binding site is situated distal to the target CD147Ex segment (i.e. on the opposite side of the molecule), preventing direct interaction of the proteins to the solid surface. In addition, the BCCP domain acts like stalk for protruding the CD147Ex segment toward the solution, allowing efficient exposure of the target domain to the tracer molecule (e.g. CD147 mAbs), or B cells in case of being trapped

on the streptavidin beads and employed as an immunogen. Moreover, protein immobilization via biotin-avidin/streptavidin interaction was proven to be superior to the passive hydrophobic adsorption by Scholle et al. who found that phage-displayed peptide ligand derived from the affinity panning against biotinylated protein-immobilized streptavidin beads only binds to the target when it is immobilized on streptavidin, and not when adsorbed to the plastic microtiter wells (Scholle et al., 2004). This observation suggests that the target protein loses its binding properties (i.e. denature, inaccessible binding surface, etc.) when affixed to plastic, the phenomenon that has been reported previously (Butler et al., 1993).

Purification process is known to be the most cumbersome step after obtaining the recombinant proteins. Normally, affinity chromatography using column conjugated with a specific target for capturing the fusion tag domain is required. However, certain conditions which facilitate the binding or elution of the fusion proteins (e.g. extreme pH, denaturing condition, or high salt concentration) may cause an adverse effect to the purified proteins (Terpe, 2003). Moreover, the procedures of the purification by affinity column are rather complicated. To overcome these problems, in this study, the streptavidin-coated magnetic beads were used to sort the CD147Ex-BCCP from bacterial crude extract. By using external magnetic field, the magnetic beads anchoring CD147 via biotin-streptavidin specific reaction were simply separated from the solution mixture. Since the single biotinylation site is situated at the opposite side of the CD147Ex domain, this allows favorable immobilizing the target domain in a defined orientation, in this case, toward the solution. In addition, this one-step method needs less time and cost consuming in comparison to other expression systems which require sophisticated affinity

purification steps. In an attempt to produce antibody responses to CD147, the capacity in carrying the target domain CD147Ex of the magnetic beads was demonstrated by immunofluorescence and flow cytometry. The fusion protein-captured magnetic beads showed strong fluorescent intensity after being stained with CD147 mAb M6-1D4, but not for the uncoated beads. This indicated the immobilization of CD147Ex-BCCP fusion protein on the magnetic beads, which were then employed as an immunizing agent.

The antibody response generated in the immunized mice was determined by indirect ELISA in which the optimal dilution of cell culture supernatant containing recombinant CD147-hIgG fusion protein was coated on the wells to exclude the activity of anti-streptavidin and anti-BCCP antibodies. The mice sera were two-fold serially diluted and the pre-immunized serum of each mouse was used as a cut-off titer. The specific antibody activity was early detected (2 weeks after 1^{st} immunization, titer $1.6x10^4$) and the significant titer ($5x10^5$) of anti-CD147 antibodies was observed in all immunized mice. In contrast, there was not significant binding of mice antibodies to the wells coated with supernatant containing CD2-hIgG. Because of using the same preparation procedures as CD147-hIgG, the nearly undetectable signal from the wells coated with CD2-hIgG excluded the binding of mice antibodies to irrelevant molecules, such as IgG part, secreted cellular proteins or components of serum used in culture medium. In addition, the antibody level of all mice was maintained for a long period of time (5 months after the last immunization). Furthermore, the antibody activity against the native membrane-bound CD147 was evaluated by immunofluorescence and flow cytometry. According to the FACS histogram, the 6^{th} week serum from the representative immunized mice specifically

reacted with CD147-expressing BW5147 cell, but not for CD298-expressing or normal BW5147 cells, whereas the pre-immunized serum did not react to any cell types. To further assure the specificity of the binding, the protein extracts from those three cell lines were separated by SDS-PAGE and blotted onto PVDF membrane. Regarding the comparable amount of protein in all of the cell lysates, the expected protein band of mature CD147 (range from 45-55 kDa, variable size due to the degree of glycosylation) was visualized only in the CD147-expressing cell lysate after being probed with the immunized serum, but not with the pre-immunized serum. The same size of reactive band was also observed after probing with CD147 mAbs, confirming the specificity of the obtained mouse antibodies to CD147. These results reflect the excellence of our immunogen over the conventional whole cell immunization, which always resulted in development of antibodies to cellular proteins other than the target molecule. This newly developed immunization strategy efficiently induced the production of highly reactive and specific mouse polyclonal antibodies, which were demonstrated to be able to strongly react with CD147 both in native and denatured form.

According to the previous data obtained in our laboratory, BCCP fusion protein-captured streptavidin beads approach has been successfully used to induce antibody responses to the target protein survivin, which failed to trigger immune responses when displayed on the filamentous phage (unpublished data). Indeed, phage display technique has often been demonstrated to be an excellent system in delivering foreign proteins to the immune system (Rao et al., 2003; Frenkel et al., 2004; Solomon, 2005). However, phage particle contains several structural proteins which somehow may quench the recognition of the displayed proteins by the immune cells,

especially when the target proteins are low immunogenic. Regarding single biotinylation site proximity to the C-terminal, the BCCP stalk stretches the CD147Ex portion out from the bead surface. This allows the B cells to respond against CD147Ex B cell epitopes more efficiently. In addition, the particulation of immunogen in this way should trigger the immune responses more potent due to better antigen-presenting cell induction compared to the purified soluble form. The small particles like magnetic beads captured with the target proteins may be the potential carrier for proteins that are naturally non-immunogenic or do not expose to the immune system, such as cytoplasmic domain of membrane protein or hydrophobic region. Thus, the biotinylated antigen-coated streptavidin magnetic beads represent the powerful immunizing agent and could be useful in challenging the poor immunogenic target proteins.