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Establishment of a signal peptide with cross-species compatibility for functional antibody expression in both *Escherichia coli* and Chinese hamster ovary cells

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ABSTRACT

Signal peptides are short peptides located at the N-terminus of secreted proteins. They characteristically have three domains; a basic region at the N-terminus (n-region), a central hydrophobic core (h-region) and a carboxy-terminal cleavage region (c-region). Although hundreds of different signal peptides have been identified, it has not been completely understood how their features enable signal peptides to influence protein expression. Antibody-derived signal peptides are often used to prepare recombinant antibodies expressed by eukaryotic cells, especially Chinese hamster ovary (CHO) cells. However, when prokaryotic *Escherichia coli* (*E. coli*) are utilized in drug discovery processes, such as for phage display selection or antibody humanization, signal peptides have been selected separately due to the differences in the expression systems between the species. In this study, we successfully established a signal peptide that enables a functional antibody to be expressed in both prokaryotic and eukaryotic cells by focusing on the importance of having an Ala residue in the c-region of the signal sequence. We found that changing Ser to Ala at only two positions significantly augmented the anti-HER2 antigen binding fragment (Fab) expression in *E. coli*. In addition, this altered signal peptide also retained the ability to express functional anti-HER2 antibody in CHO cells. Taken together, the present findings indicate that the signal peptide can promote functional antibody expression in both prokaryotic *E. coli* and eukaryotic CHO cells. This finding will contribute to the understanding of signal peptides and accelerate therapeutic antibody research.
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1. Introduction

Antibodies are major therapeutic proteins used for cancer, allergies and other unmet medical needs. Fabs are the functional antibody domains used for antigen binding, and are emerging therapeutic proteins [1]. The protein expression efficiency is a key area of research and development that need to be improved for the generation of therapeutic antibodies and Fabs. Both prokaryotic and eukaryotic cells have been utilized in the various drug discovery processes such as phage display selection [2] or humanization [3] to synthesize the lead monoclonal antibody candidate. *Escherichia coli* has been a dominant prokaryote host used for economical manufacturing processes [4], and it has been successfully used to produce clinically available proteins such as insulin [5], growth hormone [6], G-CSF (granulocyte colony-stimulating factor) and some other therapeutic proteins [7]. On the other hand, CHO cells have been used as eukaryotic cells to generate recombinant proteins which need post transcription modifications for the therapeutic activities, such as EPO (Erythropoetin) [8] and numerous monoclonal antibodies [9].

The N-terminus of synthesized secretory proteins contains a peptide sequence consisting of 15–30 amino acids referred to as a signal peptide [10]. Signal peptides control the entry of virtually all proteins into the secretory pathway, in both eukaryotes and prokaryotes [11]. The domain of signal peptides from various proteins are commonly described as a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region [12]. These are cleaved off after the proteins are translocated through cellular membrane. The cleavage sites usually conform to the established rule for residues at positions −3 and −1 from the cleavage site in prokaryotes. The rule states that small and neutral residues (Gly, Ser, Ala) at positions −3 and −1 may enhance the rate of the cleavage process by bacterial signal peptidase I (Lep) [13–15]. Comparative studies of signal peptides cleaved by signal peptidases I and II suggested that these are different for the c-region. A well-conserved signal sequence, Leu-Ala-(Gly,Ala) followed by Cys, defines the bacterial signal peptidase II (Lsp)

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cleavage site [16]. In terms of length, signal peptides from eukaryotes tend to have slightly shorter n-, h-, and c-regions than signal peptides from Gram-negative bacteria. Conversely, Gram-negative bacterial signal peptides have shorter regions than Gram-positive bacteria [17]. The structural homology among those signal peptides is such that they can similarly work when they are transferred to another species, but usually result in a lower level of secretion.

Although hundreds of different signal peptides have been identified and compared structurally, most of the previous studies on the function of signal peptides have been limited to bacteria and yeast. Signal peptide modification of human IL-2 was investigated on correlation between the sequence properties and rate of endostatin or alkaline phosphatase protein secretion in a human malignant cell line, MDA-MB-435 [18]. This study revealed that basicity and hydrophobicity at h-region were key features in human signal peptides. Cross-species compatibility has been demonstrated separately in examples such as rice α-amylase expressed in *Saccharomyces cerevisiae* [19], human CD23 expressed in a baculovirus system with a signal sequence derived from a gram-positive bacterium, Staphylococcal protein A [20]. However, the compatibility of mammalian signal peptides with bacterial hosts remains unknown, which leads to poor cleavage rates and low yields of secreted proteins, especially antibodies. On the other hand, bacterial expression systems have been utilized during the drug discovery process and the findings of studies performed to develop these systems also support an industrial manufacture process using *E. coli* or CHO as a host cell [21].

To this end, we tested a range of *E. coli* and mammalian signal peptides on the Fab expression in *E. coli* or the antibody expression in CHO cells, but these signal peptides did not show compatible expression at all. Amino acid residues at positions −3 and −1 in the c-region were previously reported to be important for the recognition and cleavage of signal peptidase I in *E. coli* [22]. In particular, the residue at position −1 must be a small side chain such as Gly, Ser or Ala to allow for efficient protein secretion. In this study, we tested a signal peptide derived from a mouse antibody because this signal peptide has three continuous Ser residues at the C-terminal. Even though this signal sequence was fused to the N-terminus of Fab, we failed to express Fab in *E. coli*. Next, we precisely examined an amino acid residue substitution from Ser to Ala at both positions −3 and −1 in the c-region and found that Fab expression could be significantly enhanced by this modification in *E. coli*. We also found that this altered signal sequence retained the ability to induce the expression of the functional antibody in CHO cells. These data clearly indicated that we succeeded in creating a compatible signal peptide to express a functional antibody in both prokaryotic and eukaryotic cells.

2. Materials and methods

2.1. Cloning and expression of recombinant proteins

The genes encoding a humanized anti-HER2 Fab (4D5) [23] were synthesized for insertion into an expression vector after codon optimization for each host, including signal sequences. For bacterial expression, the variable regions, including the signal peptides of anti-HER2 Fab were separately subcloned into a pFLAG-based vector (Sigma Aldrich) to prepare the promoter and a series of signal peptide derivatives. Plasmid vectors were introduced into *E. coli* strain W3110 by the heat-shock method. For eukaryotic cell expression, human anti-HER2 antibody expression plasmids were generated by cloning them into the pKANTEX93 vector [24]. These plasmid vectors were introduced into the DG44 CHO cell line by electroporation and G418-resistant clones were obtained. The supernatants were applied to 1 mL of Mabselect resin (GE Healthcare) and purified to prepare anti-HER2 antibodies. The soluble human HER2 extracellular domain fused to a His-tag was generated by polymerase chain reaction (PCR) and cloned into the pKANTEX93 vector. This vector was introduced into rat hybridoma cell line YB2/0 by an electroporation method and G418-resistant clones were thus obtained. The supernatant was added to 0.5 mL of TALON resin (Clontech) and soluble human HER2 antigen was purified.

2.2. Fermentation of E. coli expressing Fab fragment

Vector-transformed cultures of W3110 were incubated at 37 °C in Luria–Bertani broth. The pre-culture fluids were inoculated into 200 mL of Super Broth to start fermentation in a 1 L Erlenmeyer flask [25]. When the absorbance at 600 nm reached 2.0, the temperature was shifted to 20 °C and the bacterial culture continued overnight after the addition of 0.1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). After centrifugation of the culture broth, cell pellets were lysed using the B-PER extraction reagent (Thermo Scientific), the cell lysates were clarified by centrifugation (4500*g*, 20 min). After filtration (0.22-μm), the supernatant was applied to TALON resin (Clontech) and Fab fragments were purified.

2.3. Detection and quantification of secreted Fab protein

The yields of each Fab sample in the culture fluid were measured by sandwich ELISA. Flat-bottomed 96-well plates were first coated with 100 μl of 1:1000 diluted anti-human IgG F(ab)′2 fragment (Jackson Immuno Research) in carbonate-bicarbonate buffer. After being blocked with Super blocking buffer in TBS (Thermo Scientific), purified Fabs were incubated on the plates at room temperature for 1 h. After being washed with PBS containing 0.05% Tween 20 (wash buffer), the binding Fabs were detected using HRP-labeled goat anti-human kappa antibody (Southern Biotech) with the substrate tetramethylbenzidine (Wako). The reaction was stopped with the addition of 0.5 mmol/L sulfuric acid (Wako), and the absorbance at 450 nm/595 nm was measured by a Sunrise plate reader (Tecan). The total amount of Fab was calculated by a standard curve using reference samples.

2.4. Polyacrylamide gel electrophoresis (SDS–PAGE)

A total of 10 μl of purified samples were mixed with 2 μl of 6× sample buffer (Nacalai) and loaded onto Mini-PROTEAN TGX gels (Bio-rad) in running buffer. A 10 μl aliquot of SpectraTM Multicolor Broad Range Protein ladder marker (Thermo Scientific) was loaded onto the gel simultaneously. The running conditions followed a standard protocol (300 V, 25 min). To visualize the proteins after electrophoresis, the gels were stained with Coomassie Blue for 1 h and then were destained for 1 h. The destained gels were photographed using a LAS-3000 system (FUJI FILM).

2.5. Antigen binding assay

The antigen binding properties of the purified antibody or Fab were measured by ELISA. Flat-bottomed 96-well plates were coated with human HER2 antigen in TBS. After being blocked with Super blocking buffer in TBS (Thermo Scientific), the purified antibodies or Fabs were quantified and the same amount of each antibody or Fab was added to the plate. The plate was incubated at room temperature for 1 h. After being washed with TBS containing 0.05% Tween 20, bound Fabs or antibodies were detected using an HRP-labeled goat anti-human light chain antibody (Bethyl Laboratories) or an HRP-labeled goat anti-human IgG antibody (American Qualex) with the substrate tetramethylbenzidine (Wako). The

Table 1
The eukaryotic and prokaryotic signal peptide sequences.

Name	Sequence
Eukaryotic signal peptides	
A native mouse antibody (SSS)	MKLPVRLLVLMFWIPA**SSS**
Ala at position −3 (ASS)	MKLPVRLLVLMFWIPA**ASS**
Ala at position −1 (SSA)	MKLPVRLLVLMFWIPA**SSA**
Ala at positions −3 and −1 (ASA)	MKLPVRLLVLMFWIPA**ASA**
Prokaryotic signal peptides	
OmpA	MKKTAIAIAVALA**G**FATV**AQA**
PelB	MKYLLPTAAA**G**LLLLAAQP**A**M**A**

A eukaryotic signal peptide sequence selected from a mouse antibody was identified with three consecutive Ser (SSS) residues in the c-region. We altered the SSS sequence in the c-region to be ASS, SSA or ASA. The residues from position −3 to −1 relative to a cleavage site are indicated in boldface. Each h-region is indicated in underlined text. In the middle of h-region of OmpA and PelB, the helix-breaking residue (Gly) is indicated by bold text.

reaction was stopped with the addition of 0.5 mmol/L sulfuric acid (Wako), and the absorbance at 450 nm/595 nm was measured by a Sunrise plate reader (Tecan).

3. Results and discussion

3.1. Optimization of the eukaryote signal peptide for Fab expression in E. coli

The signal peptides characteristically have certain features in common, including a basic region at the N-terminus in the n-region and a central hydrophobic core in the h-region. They also contain small and neutral residues (Gly, Ser and Ala) in the c-region in prokaryotic and eukaryotic cells. Several signal peptides, such as OmpA and PelB, actually show effective expression in prokaryotic cells. We selected mouse antibody-derived signal peptide that contains three continuous Ser residues (SSS) in c-region (Table 1). We expected this signal sequence to be suitable for Fab expression in prokaryotic cells, because this signal retains the Ser residues at positions −3 and −1 to allow for the efficient cleavage by bacterial signal peptidase I. However we found that this signal sequence was ineffective for Fab expression in *E. coli*.

We next explored alterations to this signal sequence to promote the efficient expression of Fab in *E. coli*. A previous report revealed that the presence of Ala residues at positions −3 and −1 is common in prokaryotes [26]. To confirm the importance of having Ala residue at positions −3 and −1, we focused on these positions in the signal peptides derived from the mouse antibody used in this study. We altered the three consecutive Ser residues sequence (SSS) in the c-region of the signal sequence to be Ala-Ser-Ser (ASS), Ser-Ser-Ala (SSA) or Ala-Ser-Ala (ASA) and examined the resulting Fab expression. After the Ala residue substitution of these signal peptides at positions −3 and −1, the amount of each secreted Fab in *E. coli* was measured. The sandwich-ELISA showed that a signal peptide alteration from SSS to ASA led to a major impact on Fab secretion in *E. coli* (Fig. 1A). We next purified Fabs from these *E. coli* culture broths using metal chelate affinity chromatography. The result of SDS–PAGE (under non-reducing conditions) correlated with the results of the sandwich ELISA. While ASS and SSA sequences promoted a

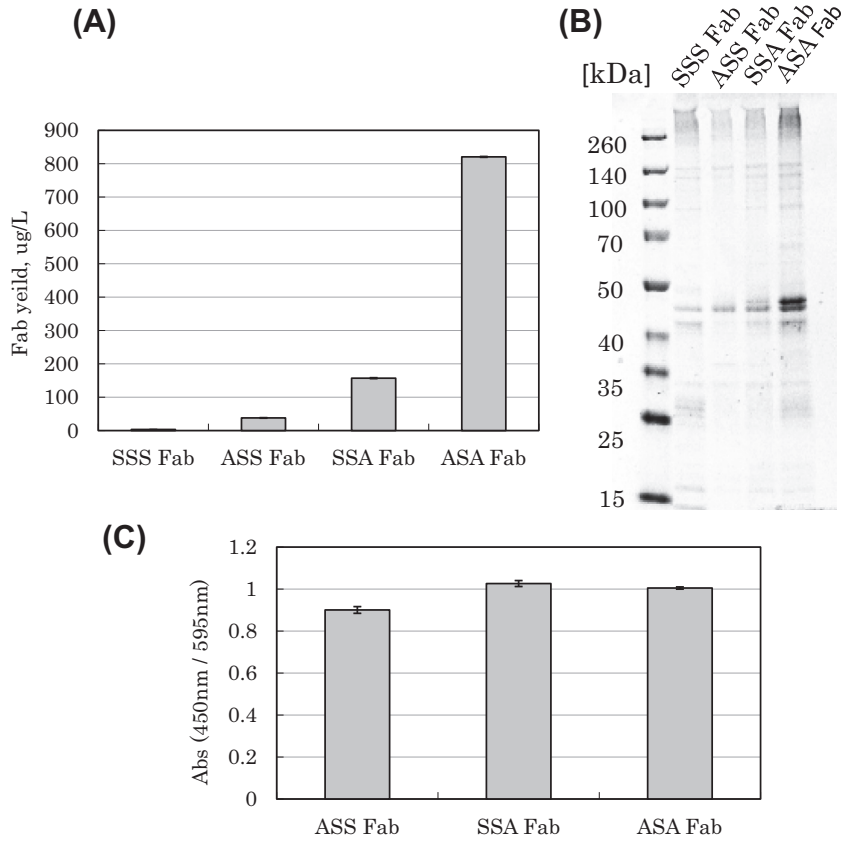

Fig. 1. The result of the measurements of anti-HER2 Fab expression and properties of the different residues at the C-terminal. The signal peptide derived selected from mouse antibody was modified focusing on the residue at positions −3 and −1 in c-region. Fabs were prepared by culturing *E. coli* W3110 cells in 1 L Erlenmeyer flasks. SSS Fab, ASS Fab, SSA Fab or ASA Fab stands for anti-HER2 antigen binding fragment whose three residues at C-terminal is SSS, ASS, SSA or ASA. (A) The total amount of each purified Fab from 200 mL culture broth was quantified by sandwich-ELISA after metal chelate affinity chromatography. (B) Purified Fab samples were analyzed by SDS–PAGE (under non-reducing condition). (C) Fab binding ability was investigated by ELISA using HER-2 antigen immobilized to flat-bottomed 96-well plates. Absorbance (Abs) at 450 nm was detected (the reference wave length was 595 nm).

slightly higher secretion than SSS, the ASA sequence showed significantly higher secretion (Fig. 2B).

Alteration of the Ser to Ala at positions −3 and −1 of the signal peptide turned out to have a significant effect on the Fab secretion in *E. coli*. An antigen binding assay using an ELISA was conducted to confirm the binding activity of each Fab to the HER-2. The ELISA showed that the ASS, SSA and ASA sequences in the c-region resulted in equivalent antigen binding activity (Fig. 2C). Although both Ala and Ser are small and neutral amino acid residues, Ala at positions −3 and −1 of signal peptide led much higher secretion of Fab protein. We speculated that this could be based on the fact that a nonpolar amino acid residue Ala at positions −3 and −1 would uniquely enhance the rate of cleavage processing by bacterial signal peptidase I (Lep) compared to polar amino acid residue Ser.

We also examined other Fab expressions in *E. coli* and succeeded in expressing all Fabs by using a signal sequence containing the ASA sequence (data not shown). The secondary structure at the cleavage junction of preproteins also plays an important role in functional protein expression. Moreover, the host strain, signal sequence in the n-region or h-region and structure of the protein can affect the efficiency of protein secretion. In fact, as shown in Table 1, the selected signal peptide never contains a helix-breaking residue (mostly Gly) in the middle of the h-region or at position −7 to −4 relative to the predicted processing site for Lep of *E. coli* [27]. PelB and OmpA do have such helix-breaking residues in the middle of the h-region. Thus we do not consider that a substitution of an Ala residue at positions −3 and −1 of a eukaryotic signal sequence is a general method that can be used to create a signal sequence for the efficient expression of all functional antibodies in *E. coli*. However, our signal sequence established in this study can express functional Fab, and can be applicable for different kinds of Fab expression in *E. coli*.

3.2. Analysis of signal peptide compatibility of monoclonal antibody expression in CHO cells

After the alteration of Ser to Ala in the c-region, the ASA sequence led to much higher bacterial expression of Fab. In terms of eukaryotic expression, a native antibody signal peptide has been used for antibody expression in CHO cells during therapeutic antibody screening. We selected a signal sequence derived from a mouse antibody because this signal peptide has continuous three Ser residues the in c-region. The tendency for an ASA sequence to occur in the c-region was found less often in eukaryotic signal peptide than gram-negative and gram-positive bacteria [28]. Therefore, the C-terminal sequence recognition by the eukaryotic signal peptidase would not be expected to be completely the same as that of the prokaryotic signal peptidase. We herein investigated the compatibility of the modified signal peptide in CHO for various applications of antibody therapeutic technologies.

In this study, the SSS and ASA sequences in the c-region were compared when HER-2 antibodies with these signal peptides were prepared by CHO cells. After these CHO clones were sorted by G418-resistance selection, secreted antibodies were purified through protein A affinity chromatography. SDS–PAGE (under reducing conditions) showed appropriate molecular weights for these antibody light and heavy chains (Fig. 2A). We confirmed that all N-terminal protein sequences of the Fab and antibody were correctly cleaved by identifying the sequences by electrospray ionization (ESI) Orbitrap (+) mass spectrometry (MS) (data not shown). The antigen binding properties of the anti-HER2 antibody were measured by ELISA. The results therefore demonstrate that the SSS and ASA sequence resulted in the equivalent antigen binding activity of the antibodies (Fig. 2B). Taken together, these finding indicate that we successfully established a eukaryotic signal peptide that promotes the compatible expression of a functional antibody in both *E. coli* and CHO cells.

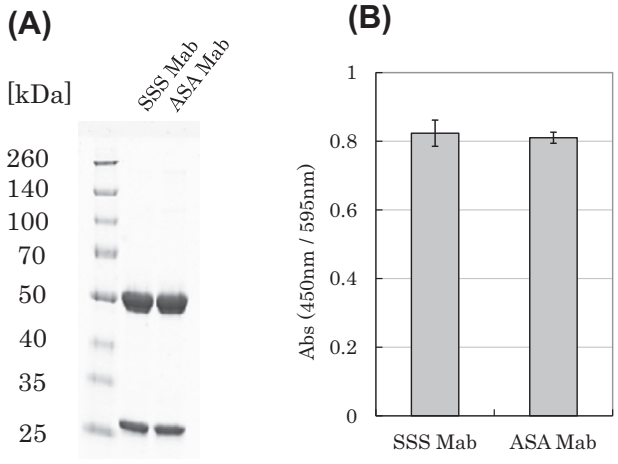

Fig. 2. The result of the analysis of anti-HER2 antibody properties of the different residues at the C-terminal. The original signal peptide derived from a mouse antibody (SSS) was compared with the modified sequence (ASA) in the c-region. The anti-HER2 antibodies were prepared by culturing the CHO DG44 clones. SSS Mab or ASA Mab stands for the anti-HER2 monoclonal antibody whose three residues at the C-terminal are either SSS or ASA. (A) The purified Mab samples were analyzed by SDS–PAGE (under reduced conditions) after the protein A chromatography. (B) The Mab binding ability was investigated by ELISA using the HER-2 antigen immobilized to flat-bottomed 96-well microplates. Absorbance (Abs) at 450 nm was detected (the reference wave length was 595 nm).

4. Conclusion

This study suggested that a eukaryotic signal peptide with Ala (as a nonpolar amino acid) at positions −3 and −1 in the c-region plays a significant role in efficient Fab expression in *E. coli*. Few previous studies have examined how their features enable eukaryotic signal peptides to influence antibody expression. We tested a signal peptide derived from a mouse antibody with an SSS sequence in the c-region. After we changed the Ser to Ala at positions −3 and −1 in the c-region, we found that an ASA sequence dramatically improved the Fab expression in *E. coli*. Compared with the ASS and SSA sequences, the ASA sequence showed significantly increased secretion efficiency. Furthermore, these alterations still allowed the signal peptide to retain the features to induce the expression of a functional antibody in CHO cells. This is a unique report on a eukaryotic signal peptide artificially established for compatibility with prokaryotic cells. This signal peptide could contribute to genetic engineering for various therapeutic antibody applications such as phage display, humanization and antibody production. Furthermore, these findings may be exploited to create novel signal peptides that greatly enhance the functional antibody expression in both prokaryotic and eukaryotic cells.

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