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In vitro selection of bispecific diabody fragments using covalent bicistronic DNA display

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ABSTRACT

Bispecific antibodies with two different antigen-binding sites have been widely used for a variety of medical applications. The activity and stability of antibody fragments can be improved by *in vitro* evolution. Although the affinity and stability of small bispecific antibody fragments such as diabodies can be further optimized by *in vitro* display technologies, cell-free display of bispecific antibody fragments has not been reported. In this study, we applied a covalent bicistronic DNA display for the *in vitro* selection of heterodimeric diabodies. First, we confirmed the antigen-binding activities of a diabody synthesized by an *in vitro* transcription and translation system. However, when we performed DNA-display selection of a model diabody library in a proof-of-principle experiment, no enrichment of the diabody gene was observed, likely due to a low yield of the diabody heterodimer. To overcome this issue, we introduced cysteine residues at the V_H-V_L interface of the diabody heterodimer. Using the disulfide-stabilized diabodies, we successfully enriched the diabody gene from a model library. Our results indicate that the covalent bicistronic DNA display technique could be useful for improving the stability and affinity of bispecific diabody fragments.

1. Introduction

Bispecific antibodies (bsAbs) are engineered immunoglobulin molecules with two different antigen-binding sites [1]. Because of this unique property, bsAbs are used for a variety of medical applications [1–5], such as cancer immunotherapy by bsAb-induced crosslinking of immune cells with target cancer cells [6], the delivery of therapeutic bsAbs to the brain by transferrin receptor-mediated transcytosis [7], simultaneous binding of a bsAb to factor IXa and factor X to mimic the VIII coagulation factor with hemostatic activity [8], and binding of a bsAb to epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor type I (IGF-1R), both frequently overexpressed in human tumors, to enhance Fc-mediated cellular cytotoxicity by reducing IGF-1R internalization [9].

The difficulties in preparing bispecific IgG by the co-expression of two different IgG fragments have been largely overcome by employing rational design strategies to introduce 'knobs-into-holes' mutations, disulfide bonds, and salt bridges [10]. In addition, small bsAb fragments, such as diabodies and tandem single-chain Fv fragments (scFv) [11,12], have emerged as attractive alternatives. These molecules retain the target specificity of whole IgG but can be produced more economically in *Escherichia coli* and can be further optimized by *in vitro* display technologies [13,14].

Although phage display is the most popular display technique and is the primary method for *in vitro* antibody [15] and bsAb [16] selection, it requires antibody expression in *E. coli* and thus comes with limitations derived from the use of living cells. Therefore, several cell-free display technologies which depend on *in vitro* transcription and translation (IVTT) have been developed and applied to the selection of antibody fragments (*e.g.*, ribosome display of scFv [17], V_H/K fragments [18], V-like domain [19], V_{HH} [20] and Fab [21]; mRNA display of scFv [22], Fab [23] and V_H/V_L [24]; and DNA display of single-domain antibodies [25,26] and Fab [27]). However, to our knowledge, there has been no report on the cell-free display of bsAb fragments, although an example of mRNA display of a bispecific fibronectin scaffold has been reported [28].

Abbreviations: bsAb, bispecific antibody; scFv, single-chain Fv; taFv, tandem scFv; dsDb, disulfide-stabilized diabody; CDR, complementarity-determining region; FR, framework region; IVTT, *in vitro* transcription and translation; PURE, protein synthesis using recombinant elements.

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We describe here for the first time the *in vitro* selection of bsAb fragments using a cell-free display technology.

2. Materials and methods

2.1. DNA construction

All template DNA encoding bsAbs were constructed as described in Supplementary Methods.

2.2. In vitro transcription/translation

IVTT was performed by using PURExfrex 1.0 (Gene Frontier) containing DS supplement (Gene Frontier) and a final concentration of 1–5 nM of each template DNA for 1–3 h at 37 °C or 5–7 h at 29 °C. Proteins were detected by Western blot using an anti-FLAG-tag mouse monoclonal antibody or an anti-Myc-tag (9B11) antibody (Cell Signaling Technology) as the primary antibodies, and HRP-conjugated anti-mouse IgG (GE Healthcare) as the secondary antibody, with ImmunoStar LD (Wako) as a luminescent substrate, which was detected by a C-DiGit chemiluminescent Western blot scanner (LI-COR).

2.3. ELISA

A streptavidin-coated 96-well microplate (Nunc) was incubated with 1 μM biotinylated fluorescein (Sigma) or biotinylated insulin (Sigma) in 100 μl TBST on a plate shaker at room temperature for 1 h, and then washed five times with 100 μl TBST. The resultant antigen-immobilized plate was incubated for 1 h with 100 μl of a blocking buffer (Roche) on a plate shaker, and washed five times with 200 μl TBST. After the addition of the IVTT products in 100 μl TBST, the plate was incubated for 1 h on a plate shaker and washed five times with 200 μl TBST. The plate was incubated for 1 h with a HRP-conjugated anti-FLAG antibody (Sigma) or an anti-Myc-tag (9B11) mouse monoclonal antibody in 100 μl TBST, and washed five times with 200 μl TBST. When the anti-Myc-tag antibody was used, the plate was further incubated for 1 h with the HRP-conjugated anti-mouse IgG and washed five times with 200 μl TBST. After the addition of 100 μl ELISA POD substrate (Nacalai Tesque), the reaction was stopped by 100 μl of 5 M sulfuric acid (Nacalai Tesque), and the absorbance at 450 nm of each well was measured with a Safire microplate reader (Tecan). Student's *t*-test was used for all statistical analysis.

2.4. DNA display selection

DNA display was performed as previously described [27], with some modifications. Emulsions were prepared by stirring 45 μl of PURExfrex 1.0 and 5 μl of DS supplement with 50 pM template DNA labeled with benzylguanine into 950 μl of mineral oil containing 0.45% Span 85 and 0.05% Tween 20 for 1.5 min at 4 °C. The emulsions were incubated at 37 °C for 3 h in a thermostatic bath for IVTT. An 800 μl aliquot of the top layer was collected and mixed with 240 μl of TBST containing 1% protease inhibitor cocktail and 10% BSA, and then centrifuged at 20,000 × *g* for 20 min at 4 °C. The aqueous layer from the bottom was recovered, washed with 1 ml of mineral oil, and centrifuged at 20,000 × *g* for 8 min at 4 °C. From the purified aqueous layer, an 180 μl aliquot was recovered, mixed with 20 μl of sonicated salmon sperm DNA, and used for affinity selection.

Fluorescein or insulin-immobilized beads were prepared by adding 100 pmol of biotinylated fluorescein or biotinylated insulin (Sigma) in 25 μl TBST, respectively, gently mixing for 1 h at 4 °C and washing twice with 200 μl TBST. The beads were blocked by gently mixing them with 100 μl blocking buffer for 1 h at 4 °C. The solutions were removed, and the DNA-displayed diabody described above was added to the beads and mixed for 1 h at 4 °C for binding. The beads were washed with 300 μl TBST five times and mixed with 50 μl PCR buffer. The selected DNA was amplified by PCR with Phusion DNA Polymerase (Thermo) using SP6-F and T7R primers (Supplementary Table S1) and analyzed by agarose gel electrophoresis.

3. Results and discussion

3.1. Cell-free synthesis of bispecific antibodies

To evaluate the *in vitro* selection of bsAbs, we chose fluorescein and insulin as two model antigens because we previously succeeded in the cell-free display selection of anti-fluorescein (αFlu) antibody fragments [27,29] and anti-insulin (αIns) scFv [30]. We constructed DNA that encodes for several formats of αFlu/αIns bsAbs, such as diabody and tandem scFv (taFv), and investigated the binding activity of the cell-free synthesized bsAb for each format. A diabody is a heterodimer of heterogeneous single-chain antibodies (Fig. 1A). Because it has been reported that the domain order of V_H and V_L in each fragment dramatically changes the binding activity [31,32], we tested all four possible formats of the diabody with different domain orders (Fig. 1AB; Db1/2, Db1/3, Db4/2 and Db4/3). TaFv is another format of bsAb in which two scFvs against two antigens are joined in tandem through a flexible linker (6 aa; STDGNT) [11]. We constructed two taFvs with different orders of αFlu-scFv and αIns-scFv (Fig. 1AB; αFlu-Ins-taFv and αIns-Flu-taFv).

IVTT of the bsAb fragments was performed with the PURE system [33]. The diabodies were prepared by co-IVTT of two mixed template DNA fragments; *e.g.*, F_H-I_L 1 and I_L-F_H 2 were used for synthesis of Db1/2 (Fig. 1B). TaFvs and scFvs (control) were synthesized from each single DNA template. We confirmed binding activity of the synthesized αFlu/αIns bsAbs by ELISA (Fig. 1C). Fluorescein binding was detected for both diabodies (Db1/2 and Db4/2) and taFvs, while the insulin binding was detected for only the diabodies (Db1/2, Db1/3 and Db4/2). In addition, total fluorescein binding for the taFvs was lower than that for the original αFlu-scFv (control). These results indicated that the diabody format is more suitable for the construction of αFlu/αIns bsAb in comparison with the taFv format, and that, among the four diabody formats, Db4/2 (in which both components are in the V_L-V_H order) revealed the highest binding activity. A similar trend was previously observed for diabodies specific for other antigens [32]. Therefore, we decided to choose Db4/2 for *in vitro* bsAb selection in our subsequent experiments.

3.2. Covalent bicistronic DNA display of diabodies

In a previous study, we developed a method of bicistronic DNA display and performed *in vitro* selection of heterodimeric Fab fragments linked to their encoding DNA with two ORFs [27]. In the present study, we combined this method with a covalent DNA display [34] and applied it to the *in vitro* selection of heterodimeric diabody fragments (Fig. 2).

We constructed a new Db42 DNA with two ORFs: the first ORF encodes the I_L-F_H 4 fragment fused with O^6-alkylguanine DNA alkyltransferase (SNAP-tag), and the second ORF encodes the F_L-I_H 2 fragment (Fig. 2A). We confirmed the expression and binding activity of the SNAP-tagged diabody by ELISA for both antigens (data not shown). However, when we performed the *in vitro* selection of a mixture of benzylguanine-labeled Db42 DNA (positive control) and unrelated DNA (negative control) by the covalent bicistronic DNA

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