Methods for rapid identification of a functional single-chain variable fragment using alkaline phosphatase fusion

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INTRODUCTION

Recombinant single-chain variable fragment (scFv) antibodies offer several advantages over mouse monoclonal antibodies (mAbs) for use in biotechnological applications due to their availability for genetic manipulation, which allows fusion of effector proteins such as fluorescent proteins or toxins (1, 2). Antibody libraries, with their wide range of antigen-binding specificities, have increasingly been a source of specific recombinant antibody fragments via phage display technology (3-5). However, there remains great interest in cloning and sequencing the functional VH and VL domains of a hybridoma-derived monoclonal antibody (mAb) in order to determine their contribution to antigen binding. Isolation of functional variable domains is also valuable for the rescue of unstable and/or valuable hybridoma cell lines that otherwise could be lost during long-term storage.

Unfortunately, genetic PCR amplification of functional VH and VL genes from hybridoma cell lines is somewhat complicated due to the presence of aberrant mRNAs from rearranged, non-functional heavy and light chain genes (6). It is highly likely that these non-functional VH and VL genes are more abundantly amplified than their functional counterparts due to the use of degenerate PCR primer sets, or further by mismatches and PCR errors that generate nonsense point mutations and out-of-frame clones (7, 8). Therefore, it is critical to confirm the binding specificity of the recombinant antibody protein, whose gene is cloned into an expression vector, to determine how it compares with that of the parental monoclonal antibody. This was accomplished by utilizing an experimental approach with high selection power for the identification of E. coli clones that express functional scFv or Fab fragments. So far, phage display technology (9, 10) and colony lift assays (11) have been successfully applied. Nonetheless, phage display technology suffers from technical complications that constrain its routine use (12-14), whereas colony lift assays are somewhat problematic due to a high frequency of false positives (15, 16).

To develop a simple, straightforward methodology that quickly identifies functional antibody variable genes from hybridomas, we attempted to utilize a scFv-phoA construct that had been previously successful in generating an immuno-detective tool (17, 18). Thus, a hybridoma-producing mouse IgG specific for branched-chain keto acid dehydrogenase (BCKD)-E2 (19) was used to generate a scFv-AP fusion construct, which was then transfected and expressed in E.coli. Subsequent AP-based screening clearly identified scFv-Ab specific for BCKD.

RESULTS AND DISCUSSION

Cloning scFv genes into the pPhoHis vector

Recombinant antibodies, composed of the antigen-binding fragments of whole antibodies, have numerous applications. However, correctly cloning the genes that encode antibody variable domains from a monoclonal hybridoma is not easy due to aberrant immunoglobulin transcripts, especially for kappa light chains. For instance, Kipriyanov et al. (20) reported that for the HD37 hybridoma, which produces antibodies...
against human CD19 antigen, more than half of V₅ genes obtained by PCR amplification originated from the myeloma cell line. Therefore, complicated methods such as phage display technology or colony lift assays are usually applied for the selection or identification of functional antibody variable domains from a monoclonal hybridoma.

The scFv-AP fusion protein is known to retain the functionality of scFvs without affecting antigen-binding affinity. This enables it to be applied directly to an ELISA without the use of chemically-conjugated secondary antibodies, allowing detection of nanogram concentrations of antigen (17, 18). Furthermore, the bivalency of scFv-AP fusion proteins increases the detection sensitivity of antigen-specific binders over that of their scFv counterparts in ELISAs (21). Therefore, we assumed that the scFv-AP fusion protein may provide a simple, straightforward alternative method for identifying functional antibody fragments from a hybridoma.

To prepare a cloning vector suitable for our experimental design, a modified version of the pQUANTabody vector was prepared. Briefly, a (His)₆-tag was attached to the 3' end of an in-frame phoA gene within the pQUANTabody vector, which also contains Sfi I and Not I restriction endonuclease cutting sites for scFv gene cloning, in order to facilitate purification of scFv-phoA fusion molecules as described in Materials & Methods. The resulting vector was named pPhoHis (Fig. 1). The VH and VL genes were obtained from the 2H5A12 hybridoma secreting anti-BCKD-E2 IgG mAb by PCR amplification using primers specific for antibody variable regions. Next, assembly PCR was performed to link the VH and VL genes to scFv genes. Pfu polymerase was used to increase the fidelity of PCR amplification (22). The resultant scFv DNA product was cloned into

**Fig. 1.** Schematic diagram of the expression vector construct for scFv-AP fusion molecules.

**Fig. 2.** Identification of E. coli clones expressing anti-BCKD-E2 scFv-AP fusion proteins by ELISA. (A) The culture supernatants from 48 E. coli clones were harvested and incubated in ELISA plates coated with BCKD-E2 and GST protein for 1 h. pNPP was used as a substrate and the binding signal was analyzed at OD₄₅₀ nm. (B) BCKD-E2, PDC-E2, MHSP 65, TNF-α and IDS protein were used to coat each well of the ELISA plates, which were blocked with 3% milk solution. The culture supernatant containing scFv-AP antibodies from clones #21, #46 or #47 was added to each well of the plate. Data represent the average of three experiments ± standard deviation.
the pPhoHis vector according to standard cloning procedures, followed by electroporation into high-efficiency *E. coli* ElectroTen Blue strain.

**Identification of *E. coli* clones expressing functional anti-BCKD-E2 scFv-AP fusion molecules**

In total, 48 *E. coli* colonies were randomly picked after transformation of electrocompetent *E. coli* ElectroTen Blue cells. This was followed by antibiotic selection and preparation of supernatants from the *E. coli* culture in the presence of IPTG to induce soluble scFv-AP fusion molecules. An ELISA was performed to identify *E. coli* clones that produce scFv-AP fusion proteins reactive to BCKD-E2. The results showed that 12 out of 48 *E. coli* clones produced ELISA binding signals of OD$_{450}$ nm > 0.4, indicating that 25% of clones possess significant BCKD-E2-specific binding reactivity greater than the negative control antigen, GST (Fig. 2A).

The frequency of antigen-specific recombinant clones seems extremely high considering our previous experience in which the scFv format failed to produce any positive binders recognizing BCKD-E2 from a total of 288 *E. coli* clones tested (data not shown). Similarly, when using the scFv format with the hybridoma cell line T84.66, which produces anti-CEA mAb, the frequency of positive scFv clones was less than 0.1% of those in the ELISA (data not shown). Identical PCR conditions were used to obtain scFv genes from the anti-BCKD-E2 and anti-CEA hybridomas. Considering this, antigen-specific positive recombinant clones seem to increase in frequency by at least 250-fold when a scFv-AP fusion format is used instead of a scFv format. Admittedly, it is not conclusive whether this increase in frequency comes directly from the variable recombinant antibody formats or instead is simply a reflection of the different hybridoma cell lines. Nonetheless, it is clear from our results that the scFv-AP format provides more advantages than the scFv format in identifying functional recombinant antibody fragments from a hybridoma. Additional support for this is provided by previous studies demonstrating that the bivalent scFv-AP fusion molecule produces more sensitive binding signals than its original monovalent scFv counterpart (21). One explanation is that *E. coli* produces more functional forms of scFv fused with bacterial AP than any other modified form (23), which is in accordance with the observations in this study.

Among the twenty positive scFv-phaA clones, clones #21, #46 and #47 displayed prominent binding signals to BCKD-E2 and were therefore subjected to additional ELISAs to confirm their binding specificities using BCKD-E2 and four irrelevant protein antigens, PDC-E2, MHSP-65, TNF-α and IDS (Fig. 2B). The ELISA results showed that none of the three scFv-phaA clones reacted with the negative control antigens, thereby confirming the clones specific bind to BCKD-E2. Although the data is not shown, the parental 2H5A12 anti-BCKD-E2 IgG mAb also reacts with BCKD-E2 only and not to any other proteins tested. Specific binding of the scFv-AP fusion proteins to the BCKD-E2 antigen was further tested by western blot using total cell lysates of the BCKD-E2-expressing *E. coli* clone. It was found that clones #21, #46 and #47 were reactive to recombinant BCKD-E2 protein (~56 kDa in size) only and not to any other bacterial proteins, similar to the binding reactivity of parental 2H5A12 anti-BCKD-E2 IgG mAb (data not shown).

**Characterization of the anti-BCKD-E2 scFv-AP fusion proteins**

To determine the deduced amino acid sequences of the variable antibody genes, DNA sequencing analysis of the VH and VL genes in clones #21, #46 and #47 was performed. The results showed that all three clones are identical to each other except for the single anti-BCKD-E2 scFv-phaA clone (#46), which we named 2H5scFv-PH. Amino acid sequences and CDRs of the VH and VL domains of 2H5scFv-PH are presented in Fig. 3. A BLAST search of the NCBI protein bank found that the VH domain of 2H5scFv-PH shares 87% homology with the VH of YsT9.1, which is specific for the Brucella A cell wall polysaccharide antigen. Furthermore, the VL domain of 2H5scFv-PH shares 94% homology with the VL of Pfa2, which is specific for amyloid-β (Aβ) (24). Interestingly, the light chain CDR sequences of 2H5scFv-PH and Pfa2 are almost identical except for...
for one amino acid residue difference in the CDR-L2, thus indicating the light chain may not play a significant role in antigen recognition (25).

Finally, to confirm whether the scFv-AP fusion molecule recognizes a native form of BCKD-E2, we performed immunohistochemical staining (26, 27) of NIH/3T3 cells using the E. coli culture supernatant of 2H5scFv-PH (Fig. 4). The microscopic image at ×200 magnification shows that 2H5scFv-PH stained the cytoplasmic area of the cells (Fig. 4A), which was expected since native BCKD-E2 is a mitochondrial protein. The identical staining pattern was also observed by the 2H5A12 hybridoma culture supernatant (data not shown). No significant staining was observed with anti-CEA scFv-AP molecules, NBT/BCIP substrate only (Fig. 4B), or NBT/BCIP substrate only (data not shown). These results clearly support the functionality of recombinant scFv-AP fusion protein and further demonstrate its usefulness in immunohistochemical studies. We demonstrate herein that cloning antibody variable genes in-frame with phoA provides a convenient methodology for the identification and obtaining of functional antibody variable genes from a hybridoma cell line by taking advantage of the more sensitive detection functionality of scFv-AP fusion proteins. It is clear that our approach would be quite useful for engineering mouse monoclonal antibodies and preserving valuable antibody genes from unstable hybridomas, including the generation of a valuable and readily available immunodetective antibody reagent.

MATERIALS AND METHODS

Bacterial stains and cells
The 2H5A12 hybridoma-producing mouse anti-BCKD-E2 IgG (kindly provided by Dr. M. Eric Gershwin, University of California, Davis, USA) was grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% (V/V) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin (Gibco BRL, USA) and 100 μg/ml streptomycin (Gibco BRL). NIH/3T3 (ATCC CRL-1658) cells for cell staining were grown in RPMI-1640 supplemented with streptomycin (Gibco BRL). NIH/3T3 cells for antibody reagent. 

PCR amplification and cloning of scFv genes
All DNA cloning experiments were performed according to standard procedures (28). Total RNA was isolated from 2×10⁷ 2H5A12 hybridoma cells using the RNeasy total RNA isolation kit (Qiagen, Germany). First strand cDNA was synthesized from 5 μg of total RNA using oligo dT primers using the 1st strand cDNA synthesis kit (Takara) according to the manufacturer’s protocol. Mouse heavy chain variable region genes (VH) and light chain variable region genes (VL) were PCR amplified from 1st strain cDNA using PhoA DNA polymerase (Takara) and specific primers (VH sense: 5’-GCAACTGCGCGGTGGGGTCC-3’, mVL sense: 5’-GGVACCMTGGTCACCGTCTCCTCAGGGCGGTGGCGGTGGTGTCAGCTGSWGSAGTCKG-3’, mVL anti-sense: 5’-GAGTCATTCTGCGGCCGCCCGTTTBAKYTCARKCTTKGTSCC-3’, where degeneracy denotes S = G or C, W = A or T, K = G or T, B = G, T or C, M = A or C, Y = C or T, R = G or A) under thermal profiles of 35 cycles of denaturation at 95°C, annealing at 60°C, and extension at 72°C for 1 min. Pullthrough-1 and Pullthrough-2 primers (sense: 5’-GGGGCTAGCTTTCAGCCCCAGAGCGGC-3’).

Fig. 4. Immunohistochemical staining of NIH/3T3 cells at 200× magnification. NIH/3T3 cells were prepared and immunohistochemical staining using culture supernatants of 2H5scFv-PH (anti-BCKD-E2 scFv-AP fusion) (A) or of E. coli containing anti-CEA scFv-AP fusion (B) was performed as described in Materials & Methods.
tor using a set of mVH sense and PhoAHis anti-sense primers (5’-GGGGCTCGAGTTAGTGGTGGTGGTGGTGGTGTTCGACG CCCAGAGCGGC-3’). PCR conditions were 25 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C for 1 min each. PCR products were purified on 1% agarose gels, digested with Sfi I and Xho I (Takara, Japan) and cloned into fresh pQUANTabody as described above.

Preparation of E. coli culture supernatants containing scFv-phoA fusion proteins
In total, 48 E. coli transformants were randomly picked from LB agar plates and were inoculated into each well of a sterile 96-well plate (Nunc, Denmark) containing 200 μl of 2× YT medium supplemented with 2% glucose, 50 μg/ml ampicillin and 10 μg/ml carbencillin (2× YT/GAC). Colonies were grown until approximately OD600 = 0.5. The microtiter plate was then centrifuged briefly and the culture supernatant was carefully aspirated. Bacterial cells were resuspended in 200 μl of 0.05% Tween 20 (PBST), and 50 μl of proteins was induced at 27 oC for 16 h, and culture supernatant human tumor necrosis factor-α (TNF-α) or recombinant goat anti-mouse IgG-AP (Sigma-Aldrich) were sequentially applied to the slides. After washing with three changes of PBS, goat anti-mouse IgG-AP fusion (unpublished) for 30 min at room temperature. For a positive control, the 2H5A12 hybridoma culture supernatant and goat anti- mouse IgG-AP were sequentially applied to the slides. After washing with three changes of PBS containing 20 mM EDTA and lastly with dH2O. After placing the cover slips onto the slides, cells were observed under the microscope.

DNA sequencing analysis
pPhoHis was isolated from E. coli cells producing the BCKD-E2-specific scFv-AP fusion protein using a Wizard plus SV minipreps kit (Promega, USA). A sequencing primer (5’-GCACGGCAACTCTTACGGTA-3’) complementary to the phoA signal peptide was utilized to read the scFv gene, and automatic DNA sequencing was performed by Solgent Co., South Korea.

Immunohistochemical staining of NIH/3T3 cells
Sterile glass slides were placed on culture dishes, and 106 of NIH/3T3 mouse fibroblast cells per dish were grown for 24 h in a CO2 incubator. The slides were carefully washed twice with PBS, followed by fixing of cells with 1% glutaraldehyde in PBS for 1 h at room temperature. The slides were washed again with PBS and soaked in 0.2% Triton X-100 in PBS for 20 min at room temperature. After rinsing the slides gently in PBS (four changes over 5 min), the slides were incubated with E. coli culture supernatants containing the anti-BCKD-E2 scFv-AP fusion or the anti-carcinoembryonic antigen (CEA) scFv-AP fusion (unpublished) for 30 min at room temperature in a humidified chamber. For a positive control, the 2H5A12 hybridoma culture supernatant and goat anti-mouse IgG-AP were sequentially applied to the slides. After washing with three changes of PBS for 5 min, NBT/BCIP substrate was added to the slides. To stop the enzymatic reaction, slides were rinsed with PBS containing 20 mM EDTA and lastly with dH2O. After placing the cover slips onto the slides, cells were observed under the microscope.

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