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The catalytic activity of a recombinant single chain variable fragment nucleic acid-hydrolysing antibody varies with fusion tag and expression host

Joungmin Lee [a], Minjae Kim [a], Youngsil Seo [a], Yeonjin Lee [a], Hyunjoon Park [a], Sung June Byun [c], Myung-Hee Kwon [a, b, *]

[a] Department of Biomedical Sciences, Graduate School, Ajou University, 206 World Cup-ro, Yeongtong-gu, Suwon 16499, South Korea
[b] Department of Microbiology, Ajou University School of Medicine, 206 World Cup-ro, Yeongtong-gu, Suwon 16499, South Korea
[c] Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, 1500, Kongjwipatjwi-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, 55365, South Korea

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The antigen-binding properties of single chain Fv antibodies (scFvs) can vary depending on the position and type of fusion tag used, as well as the host cells used for expression. The issue is even more complicated with a catalytic scFv antibody that binds and hydrolyses a specific antigen. Herein, we investigated the antigen-binding and -hydrolysing activities of the catalytic anti-nucleic acid antibody 3D8 scFv expressed in *Escherichia coli* or HEK293f cells with or without additional amino acid residues at the N- and C-termini. DNA-binding activity was retained in all recombinant forms. However, the DNA-hydrolysing activity varied drastically between forms. The DNA-hydrolysing activity of *E. coli*-derived 3D8 scFvs was not affected by the presence of a C-terminal human influenza haemagglutinin (HA) or His tag. By contrast, the activity of HEK293f-derived 3D8 scFvs was completely lost when additional residues were included at the N-terminus and/or when a His tag was incorporated at the C-terminus, whereas a HA tag at the C-terminus did not diminish activity. Thus, we demonstrate that the antigen-binding and catalytic activities of a catalytic antibody can be separately affected by the presence of additional residues at the N- and C-termini, and by the host cell type.

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1. Introduction

Single chain variable fragments (scFvs) are ~25 kDa recombinant antibodies in which the variable domains (V_H and V_L) are connected by a flexible peptide linker. ScFvs are of great interest to biomedical and biotechnological applications because they retain the specificity of the original antibody, can be produced recombinantly in *Escherichia coli* culture in large quantities and can be easily manipulated to generate various molecular formats such as bispecific antibodies (BsAbs) [1,2] and functional scFv fusion proteins [3,4].

Recombinant scFvs produced by bacterial and mammalian cells can differ in terms of folding properties and affinity [5], and we encountered a similar but more complex issue with autoantibody 3D8, a unique catalytic scFv antibody that can hydrolyse nucleic acids (DNA and RNA) and be internalised into a variety of live cells [6,7]. 3D8 scFv was previously generated from subcloned heavy and light chains derived from a hybridoma of an autoimmune-prone MRL-*lpr/lpr* mouse. The properties of 3D8 scFv (V_H-linker-V_L orientation) have been investigated using 3D8 scFv-$(His)_5$ secreted from *E. coli* cells, but we recently found that, when expressed in human embryonic kidney 293f (HEK293f) cells, this protein loses all DNA-hydrolysing activity, but retains DNA-binding activity. This prompted us to investigate the factors that affect the biochemical properties of 3D8, such as host cell type used for protein

List of abbreviations: AP, alkaline phosphatase; BHQ, black hole quencher; BsAb, bispecific antibody; CD, circular dichroism; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FAM, 6-carboxyfluorescein; FRET, fluorescence resonance energy transfer; HA, haemagglutinin; HEK293f, human embryonic kidney 293f; His, histidine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PAS, Periodic acid-Schiff; PBS, phosphate-buffered saline; PEI, polyethylenimine; PVDF, polyvinylidene difluoride; scFv, single chain Fv; SLS, static light scattering; ss, single-stranded; V_H, variable domain of heavy chain; V_L, variable domain of light chain.

* Corresponding author. Department of Microbiology, Ajou University School of Medicine, 206 World Cup-ro, Yeongtong-gu, Suwon 16499, South Korea.
E-mail address: kwonmh@ajou.ac.kr (M.-H. Kwon).

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expression, as well as the presence of additional amino acid residues and fusion tags at the N- and C-termini.

In the present study, we produced nine 3D8 scFv protein derivatives with distinct N- and/or C-termini by incorporating additional amino acid residues in bacterial and mammalian cell expression vectors. The resultant proteins were expressed, secreted and purified from the supernatants of *E. coli* and HEK293f cell cultures, and their DNA-binding affinity, DNA-hydrolysing activity, secondary structure, glycosylation, temperature stability and sensitivity to dithiothreitol (DTT) were compared. We observed moderate variation in DNA-binding but drastic changes in DNA-hydrolysing activity among proteins with additional amino acid residues at the N-terminus, and among those with a specific C-terminal tag sequence. These activities were also dependent on the host cell type, as demonstrated by identical amino acid sequences. This detailed comparison provides insight into the factors affecting the binding and catalytic properties of catalytic antibodies towards their antigens. Moreover, we show for the first time that the binding and catalytic activity of catalytic antibodies can be separated using different host cell types and flanking amino acid residues.

2. Materials and methods

2.1. Preparation of 3D8 scFv proteins using E. coli

To produce scFvs in bacterial cells, pIg20 vectors encoding 3D8 scFv derivatives were transformed into *E. coli* BL21 (DE3) pLysE cells (Novagen). In this vector, the V_H and V_L genes of 3D8 were connected by a $(Gly_4/Ser_1)_3$ linker following a leader signal sequence for targeting expressed proteins to the periplasm. The pIg20 vector includes an N-terminal bacterial alkaline phosphatase (PhoA) signal peptide under the control of the T7 promoter. Expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside [6], and proteins were purified from the culture supernatant in soluble form using Capto L column chromatography (GE Healthcare), based on the strong affinity of the recombinant bacterial protein L to the variable region of antibody kappa light chains, according to the manufacturer's instructions. Proteins were concentrated using a Vivaspin 20 centrifugal concentrator with a molecular cut-off of 10,000 Da (Sartorius Stedim Biotech). Protein concentration (mg/ml) was determined using the extinction coefficient at 280 nm calculated from the respective amino acid sequence.

2.2. Preparation of 3D8 scFv proteins using HEK293f cells

FreeStyle HEK293f cells (100 ml; Thermo Fisher Scientific) at a concentration of 1×10^6 cells/ml were seeded 24 h prior to transfection in a 500 ml flask (Corning Cat# 431145) to ensure that cells reached the appropriate cell density (2×10^6 cells/ml) at the time of transfection. Culturing of FreeStyle 293-F cells was performed in serum-free FreeStyle 293 media (Invitrogen Cat# 12338) at 8% CO_2 and 37 °C with shaking at 130 rpm. Plasmids KV10 and pSectag encoding 3D8 scFv derivatives were transiently introduced into 100 ml of FreeStyle 293-F cells using polyethylenimine (PEI) reagent with an average molecular weight of 25 kDa (Polyscience Cat# 23966-2). Briefly, PEI reagent (400 μg) was incubated with plasmid DNA (200 μg) at room temperature for 10 min, and then inoculated into 100 ml of cells to achieve a final PEI concentration of 4 μg/ml. After 7 days, the culture supernatant was harvested by centrifugation, and scFv proteins were purified by affinity chromatography using a Capto L column (GE Healthcare).

2.3. N-terminal sequencing

ScFv proteins were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. Each transferred sample was submitted to N-terminal protein sequence analysis by automated Edman degradation using an Applied Biosystems Procise 491 HT protein sequencer (Applied Biosystems, USA) at the Korea Basic Science Institute (Seoul center, Korea).

2.4. Enzyme-linked immunosorbent assay (ELISA)

To assay the DNA-binding activity of 3D8 scFvs, ELISA was performed as previously described [6] with slight modifications. Proteins [1 μg/ml in phosphate-buffered saline (PBS)] were reacted with a 96-well polystyrene microtiter plate (Nunc) coated with pUC57 plasmid DNA (1 μg/ml). If necessary, scFv proteins were pre-incubated with 1–30 mM DTT for 30 min at room temperature in the presence of 3 mM EDTA to block the DNA-hydrolysing activity of scFvs. Proteins bound to the wells were detected by rabbit anti-3D8 serum, which was obtained by immunizing rabbits with *E. coli*-derived 3D8 scFv-His protein, followed by alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific). The absorbance of each well was measured at 405 nm in a microplate reader. To assay the interaction between 3D8 scFv proteins and O2F3 (a mouse monoclonal anti-idiotypic IgM antibody raised against native 3D8 scFv) [8], the microtiter plate was coated with O2F3 IgM (1 μg/ml in PBS). Bound proteins bound were detected by mouse anti-His tag antibody (Abcam), followed by AP-conjugated goat anti-mouse IgG antibody (Sigma).

2.5. BioLayer interferometry

Assays for characterising the interactions between scFvs and single-stranded (ss) DNA were performed using the Octet QK^e system (Pall ForteBio Corp.). All measurements were performed at 25 °C. The DNA antigen, ss-$(dN)_{40}$ labelled with biotin at the 5′-end (bio-ss-$(dN)_{40}$) (5′-CCATGAGTGATAACACTGCGGCCAACTTACTTCT-GACAAC-3′), was synthesised by Integrated DNA Technologies Inc. The biotinylated ss-$(dN)_{40}$ DNA was immobilised at a concentration of 1 μg/ml on streptavidin sensors (Pall ForteBio) for 10 min. Typical immobilisation levels were between 0.3 and 1 nm. Sensors were stored in PBS in 96-black well microplates until needed (for up to 1 day). Sensors were quenched with 1 mg/ml BSA in PBS for 5 min, placed in wells containing various concentrations of scFvs for measuring the association (10 min) and then transferred to buffer for measuring dissociation (20 min). Kinetic constants were determined for each of the purified scFv antibodies at five concentrations between 10 nM and 200 nM by dilution in running buffer (PBS supplemented with 3 mM EDTA to block the DNA-hydrolysing activity of scFvs). All measurements were corrected for baseline drift by subtracting a control sensor exposed to running buffer only. Data were analysed using a 1:1 interaction model (fitting global, R_{max} unlinked by sensor) within ForteBio data analysis software version 9.0.0.4 (Pall Life Sciences). The dissociation constant K_D was calculated using the formula $K_D = k_{off}/k_{on}$, where k_{off} and k_{on} are the dissociation and association rate constants, respectively.

2.6. Fluorescence resonance energy transfer (FRET)-based DNA cleavage assay

The 21-nucleotide substrate conjugated with a black hole quencher (BHQ) at the 3′-terminus and 6-carboxyfluorescein (FAM) at the 5′-terminus (5′-FAM-CGATGAGTGCCATGGATATAC-BHQ-3′) was generated by M-biotech. 3D8 scFv in the reaction buffer (100 μl of TBS containing 2 mM $MgCl_2$, pH 7.2, final concentration of 250 nM) and 1 U of DNase I in 100 μl of DNase I buffer (10 mM TRIS-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6; New England Biolabs)

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