Original Article

Isolation and characterization of anti-T-antigen single chain antibodies from a phage library

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Summary **T-antigen (Galβ1-3GalNAc-Thr/Ser) also known as Thomsen-Friedenreich (TF) antigen is the core 1 structure of *O*-linked mucin type glycans. In normal epithelium, the disaccharide structure is masked by terminal carbohydrate moieties, but is uncovered in most primary and metastatic epithelial malignant tumors. For the purpose of establishing cancer diagnosis and therapeutics, anti-T-antigen antibodies were isolated from a phage library displaying human single chain antibodies. A strategy similar to the previously published method (Sakai *et al.* Biochemistry. 2007; 46:253-262) was used to screen T-antigen specific antibodies, except that a different type of glycolipid was used for panning and screening. Eleven phage clones were isolated and characterized by DNA sequencing and ELISA, which revealed 4 groups of clones with T-antigen binding activity. One single chain antibody (scFv) protein, derived from phage clone 1G11, was expressed in *Escherichia coli* and purified to near homogeneity by two column chromatographies. ELISA and surface plasmon resonance analyses revealed that the purified 1G11 scFv bound to the T-antigen moiety of the neoglycolipid used. This study not only demonstrated the validity of our previously introduced strategy employing the phage display technology in constructing human scFvs against various carbohydrate antigens, but also provided us with various scFv genes that can lead to future development of antibody-based therapeutics.**

Keywords: Human antibodies, Thomsen-Friedenreich (TF) antigen, T-antigen, single chain antibodies, neoglycolipids

1. Introduction

T-antigen (Galβ1-3GalNAc-Thr/Ser) also known as Thomsen-Friedenreich (TF) antigen is the core 1 structure of *O*-linked mucin type glycans. In normal epithelium, the disaccharide structure is masked by addition of carbohydrate chains to form branched and complex *O*-glycans. T-antigen has been identified by immunohistochemical techniques in most adenocarcinomas of the colon, breast, lung, bladder, endometrium, and ovary, as well as during embryogenesis (*1-5*). T-antigen is also known to be associated with cancer invasion (*6,7*). Anti-T-antigen human antibodies would thus provide excellent tools for not only cancer diagnosis but also cancer treatment.

While immunization with carbohydrates often leads to a primary IgM response and no response in some cases because many carbohydrates are self-antigens (*8,9*), phage display technology allows one to generate antibodies against self-antigens. This technology

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has been used mostly to generate antibodies against proteins, whereas its use for carbohydrate antigens has been limited. Previous studies utilized glycoproteins, heteroglycans, and carbohydrate-BSA conjugates as antigens to present carbohydrate-moieties for the production of anti-carbohydrate antibodies by phage display methods (*10-13*). We have adapted this phage-display technology to generate human single chain antibodies (scFvs) using neoglycolipids as antigens, and reported isolation of anti-mannotriose (M3) scFvs from a newly constructed phage library with a large repertoire as a result of CDR shuffling and VL/VH shuffling with the use of unique vector constructs (*14,15*). In the previous study, M3-dipalmitoylphosphatidyl ethanolamine (DPPE) was used as a model antigen neoglycolipid for panning and screening (*14*).

A strategy similar to the previously published method (*14*) was used to obtain T-antigen specific antibodies in this study, except that a different type of glycolipid was used for panning and screening. Galβ1-3GalNAcα-hexaethylene glycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB) contained hexaethylene glycol (E6) as a spacer that was expected to reduce non-specific binding of and to increase accessibility to phage antibodies. Its lipid anchor, bis-dodecyloxy benzamide (BDB), was also different from DPPE which was used for the previous studies. From eleven phage clones isolated and characterized, one scFv protein was expressed in *E. coli* and purified to near homogeneity by two column chromatographies. ELISA and surface plasmon resonance analyses revealed that the purified scFv bound to the T-antigen moiety of T-antigen E6-BDB. This study thus demonstrated the validity of our previously introduced strategy employing phage display technology in constructing human scFvs against various carbohydrate antigens, and provided anti-T-antigen scFv genes for future development of cancer therapeutics.

2. Materials and Methods

2.1. *Materials*

Escherichia coli strains used were the suppressor strain TG1 and the non suppressor strain Top10F' form invitrogen (Carlsbad, CA, USA). Helper phages M13KO7 were from GE Healthcare UK Ltd. (Buckinghamshire, UK). Bovine serum albumin (BSA), Human serum albumin (HSA), DPPE, and ABTS/H_2O_2 were from Roche Diagnostics (Mannheim, Germany). Of the neoglycolipids used, both T-antigen and Tn antigen-containing glycolipids (*16*) were synthesized in a laboratory at the Noguchi Institute (detailed procedures will be published elsewhere). Lacto-*N*-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (LNFPI; (Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose II (LNFPII; Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose III (LNFPIII; Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc), and lacto-*N*-difucohexaose I (LNDFHI; (Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc) were purchased from Dextra Laboratories (Reading, UK). Various oligosaccharides (LNT, LNnT, LNFPI, LNFPII, LNFPIII, LNDFHI) and DPPE were conjugated by reductive amination as described previously (*17,18*). Oxalic acid and other chemicals were from Wako Pure Chemical (Osaka, Japan). Anti-M13 antibodies were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA) and Exalpha Biologicals (Watertown, MA, USA).

2.2. *Panning procedures*

Methods for construction of phage libraries followed by recombination and generation of secondary phage scFv libraries were previous reported (*14*). The library was subjected to four rounds of panning. All rounds of panning were performed with 96-well plates coated with 500 pmol of T-antigen E6-BDB or 500 pmol of E6-BDB/well. After the 1st panning, subtraction panning was performed with 500 pmol E6-BDB/well. Forty-eight, 24, 12, and 6 wells were used for 1st, 2nd, 3rd, and 4th panning, respectively. Phage selection was basically carried out according to previously published procedures (*14*) with some modifications. Briefly, coating of wells with T-antigen was achieved by applying 50 μL of T-antigen E6-BDB (0.02 mM in methanol) premixed with E6-BDB (0.02 mM in methanol), and drying the solvent at 37°C, followed by incubation with 150 μL of 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS) and 3% BSA at 4°C, overnight. Wells were rinsed 3 times with 200 μL TBS. Fifty μL of phage suspension in TBS containing 1.4% BSA were added to wells, which were then incubated for 60 min at 37°C with continuous rotation. After wells were washed 3 times with 200 μL of TBS, bound phages were eluted by addition of 50 μL of 100 mM triethylamine and incubated at 25°C for 10 min and then neutralized by mixing with 100 μL of neutralizing solution (1 M Tris-HCl, pH 7.4, 3% BSA/TBS = 2:1, v/v) in wells, which had been treated with 3% BSA/TBS. Bound phages were further eluted by addition of 50 μL of 100 mM triethylamine and incubated at 25°C for 20 min and were recovered in the neutralization solution as described above. Eluted phages were used to infect 100 μL of *E. coli* TG1 in logarithmic growth at 37°C for 30 min. Infected bacteria, grown on LB agar plate (ø = 10 cm) (Trypton 30 g, yeast extract 20 g, NaCl 5 g, 1 M NaOH 1 mL, 1 M Mops, pH 7.0/1L) containing, 0.1% glucose, and carbenicilline (50 μg/mL) at 25°C for 48 h, were scraped from the plate using a spreader after addition of 2.5 mL of LB-10 mM Tris-HCl, pH 7.5 (SBS) per plate. One mL of this suspension was inoculated into 40 mL SBS containing carbenicilline

(50 μg/mL) and grown with shaking at 37°C for 2 h. Phages were rescued after addition of 40 μL of helper phages containing 3.5×10^9 cfu and incubated at 37°C for 1 h without shaking, followed by addition of kanamycin (25 μg/mL)/chloramphenicol (10 μg/mL) and incubated with rotation at 25°C for 48 h. Phage particles were concentrated using PEG-precipitation, and dissolved in 700 μL of TBS, 700 μL of 3% BSA/TBS, and 1 μL of Benzonase and incubated at 37°C for 1 h. After centrifugation at 18,000 × g for 5 min at 4°C, 1.5 mL of phage suspensions were recovered and used for subtraction panning. For subtraction panning, 50 μL phage suspension/well were applied to E6-BDB-coated wells, and incubated at 37°C for 1 h. Phages not bound to E6-BDB were recovered and used for 2nd, 3rd, and 4th panning which were carried out in a similar fashion to the 1st panning except for washing conditions. Bacteria picked from single colonies after 4 rounds of panning were grown in 200 μL of SBS containing carbenicilline (50 μg/mL), 2% glucose and 6% glycerol in 96 deep-well plates at 37°C for 2 days with rotation. Twenty μL of the bacterial culture were added to 200 μL of SBS containing helper phages and carbenicilline (50 μg/mL), and incubated at 37°C for 2 h without rotation. SBS/kanamycin/chloramphenicol mixture (300 μL/well) was added to the suspension culture, from which phage suspensions were prepared by incubation at 25°C for 32 h with rotation. After centrifugation at 200 × g for 15 min at 4°C, 100 μL of the supernatants were added to wells containing 100 μL of 3% BSA/TBS, incubated at 37°C for 1 h, and kept at 4°C.

2.3. *Screening of phage clones expressing scFvs directed against T-antigen E6-BDB by ELISA*

Binding ability to T-antigen E6-BDB of the bacterial supernatants containing phages was determined by ELISA. Wells of 96-well plates were coated with 500 pmol T-antigen E6-BDB and 500 pmol E6-BDB/well as described above and blocked by incubation with 150 μL of 3% BSA/TBS at 4°C overnight. Control plates were prepared as above without the antigen. Fifty μL of phage suspension were added to the wells and incubated at 37°C for 1 h. The wells were washed 10 times with 200 μL of TBS. Bound phage antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated anti-M13 antibody at 37°C for 1 h, after which they were washed 5 times with 200 μL of 0.2% Tween 20/TBS and washed 10 times with 200 μL of TBS. Peroxidase activity was detected by reaction with ABTS/H_2O_2 for 30 min and terminated with 1% oxalic acid. Absorbance was measured with a BIO-RAD plate-reader at 415 nm.

2.4. *Colony PCR and determination of DNA sequences*

Colony PCR was carried out according to the previously published procedure (*14*). Briefly, scFv genes were amplified from respective *E. coli* TG1 colonies infected with phages by PCR with a primer set (forward primer Cm-f: 5'-TGTGATGGCTTCCATGTCGGCAGAATGC T-3', reverse primer g3-r: 5'-GCTAAACAACTTTCAA CAGTCTATGCGGCAC-3'). After preheating at 94°C for 2 min, PCR was carried out using 30 cycles under denaturing conditions at 94°C for 20 sec, annealing at 60°C for 20 sec, and extension at 68°C for 1 min. After purification and confirmation in 2% agarose gel electrophoresis, the resulting scFv genes were subjected to DNA sequencing. DNA sequences of scFvs were determined using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. *Determination of carbohydrate-specificity of phage antibodies by ELISA using synthesized neoglycolipids*

Wells of a 96-well plate were coated by addition of 50 μL of LNT-, LNnT-, LNFPI-, LNFPII-, LNFPIII-, and LNDFHI-DPPE, as well as T-antigen E6- and Tn-antigen E6-BDB (500 pmol)/well and drying the solvent at 37°C, followed by incubation with 150 μL of 3% BSA/TBS at 4°C overnight. The rest of the procedures were carried out as described above for screening of phage clones.

2.6. *Expression and preparation of the 1G11 scFv protein*

The 1G11 phage-infected *E.coli* TOP10F' was cultured in 200 mL of SBS containing 0.4% glycerol, carbenicilline (50 μg/mL), spectinomycin (50 μg/mL), and 1 mM isopropyl-thio β-D-galactopyranoside (IPTG) at 25°C, overnight. Cells were collected by centrifugation at 4,000 × g for 30 min, and solubilized with non-ionic detergent Bugbuster (0.2 g whole cells/1 mL) (EMD chemicals, Inc., CA, USA). The extract was diluted 10-fold by the addition of 20 mM Tris-HCl, pH 8.0, and applied to a DEAE-cellulose column (DE52, Whatman, GE Healthcare) (ø1.5 × 28, 50 mL). The DEAE-cellulose column was equilibrated with 20 mM Tris-HCl, pH 8.0, before applying the sample. Bound proteins were eluted using a 0-1 M NaCl gradient. Fractions eluted from the column were analyzed using SDS-PAGE (4-20% polyacrylamide gel, Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions, followed by staining with Coomassie Brilliant Blue (CBB). The proteins in a duplicate SDS-PAGE gel were transferred to a PVDF membrane. After blocking with 3% BSA/PBS, the 1G11 scFv protein was detected with an HRP-conjugated anti-E-tag antibody followed by fluorescence development using an ECL detection kit (GE Healthcare). The fractions containing immunoreactive bands were analyzed by surface plasmon resonance (SPR) to determine T-antigen binding activity of the eluates.

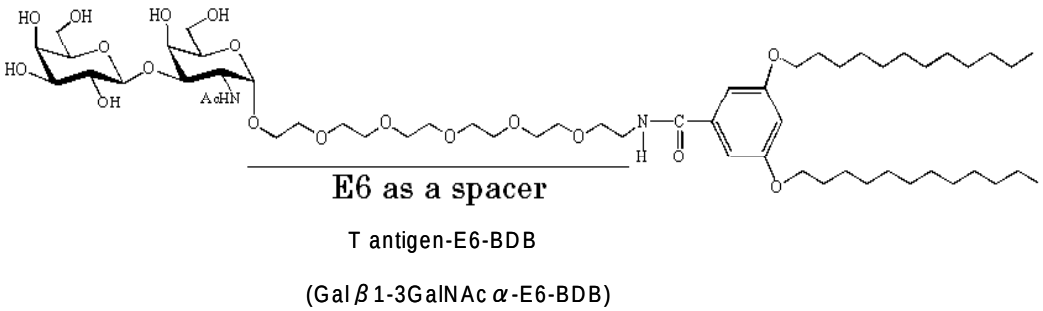

Figure 1. Structure of the T-antigen neoglycolipid used for panning and screening in this study. Galβ1-3GalNAcα-hexaethyleneglycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB) is illustrated.

Fractions #21 to #26 which showed immunoreactivity to anti E-tag and binding activity to T-antigen were combined. To this solution, urea and 200 mM imidazole buffer were added to adjust final concentrations to 2 M and 20 mM, respectively. The DEAE-cellulose eluates were then incubated with 2 mL of Ni^{2+}-Sepharose gel (GE healthcare) on a rotator, overnight at 4°C. The gel was packed in a column, from which the passed-through fractions were collected. After the column was washed with 40 mL of PBS containing 40 mM imidazole, the 1G11 scFv protein was eluted with 400 mM imidazole/PBS. Protein concentrations were determined using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) with BSA used as a standard.

2.7. *T-antigen binding analysis of scFv proteins by surface plasmon resonance*

SPR analyses were performed at 25°C. All solutions were freshly prepared, degassed and filtered through a 0.45 μm pore filter. Binding properities of DEAE-cellulose eluates were determined by SPR using BIAcore 3000 (GE Healthcare). Immobilization on a CM5 sensor chip was carried out using amine coupling at pH 4.5 for 14 min during activation according to the manufacture's instructions. T-antigen-HSA (Dextra Laboratories) and HSA (Sigma) were immobilized at 100 μg/mL onto a positive flow cell and a control flow cell, respectively. The binding analyses were carried out with 10 mM Hepes, pH 7.4, containing 150 mM NaCl and 0.005% Tween 20 at a flow rate of 20 μL/min. The specific binding of the sensorgrams was obtained by subtraction of the control flow channel sensorgram from that of the positive flow cell.

3. Results

3.1. *Screening of scFv-displaying phages against T-antigen E6-BDB*

A phage library displaying human scFvs was subjected to four rounds of panning as previously described using a neoglycolipid (*14*). The structure of the antigen neoglycolipid used in this study for panning and screening is shown in Figure 1. Of 96 clones screened by ELISA using T-antigen E6-BDB as an antigen, there were 24 positive clones with a S/N of > 3 (Table 1). Of those, 11 positive clones were confirmed to encode scFvs by PCR and DNA sequencing. DNA sequencing of scFv sequences of 11 clones revealed deduced amino acid sequences of VH and VL chains as shown in Table 2A and 2B, respectively. Those scFvs were categorized into four groups based on their amino acid sequences (Table 3). Three Group 1 clones, 1E8, 1F6, and 1H7, were found to be identical. Two Group 2 clones, 1E6 and 1F4, shared homology in their heavy chains but differed in their light chain sequences. In contrast, three Group 3 clones, 1E10, 1F1, and 1F9, showed homology only in their light chains. Two identical Group 4 clones, 1G11 and 1H11, were found to be an incomplete scFv without CDRs in their light chains. One additional clone, 1F5, did not show any amino acid sequence homology to those of other clones.

Table 1. Screening of phages displaying scFvs having affinity for T-antigen E6 BDB

	Number of clones
Screened by ELISA	96
ELISA-positive (signal/noise ratio: > 3)	24
DNA sequencing-confirmed scFvs	11

3.2. *Characterization of phages displaying scFvs screened by T-antigen E6-BDB*

Figure 2 shows specificity profiles of phage antibodies, 1E8, 1F6, 1E10, and 1G11, representing Group 1, 2, 3, and 4, respectively, as determined by ELISA using a set of neoglycolipids. A Group 4 clone, 1G11, had the highest specificity and affinity for T-antigen. In contrast, other Group 3 clones showed cross-reactivity to Tn-antigen E6 BDB and LNFPIII-DPPE. Thus, 1G11 was chosen for further characterization.

3.3. *Expression and purification of the 1G11 scFv protein*

E. coli Top10F′ infected with the 1G11 phage was

Table 2A. Deduced amino acid sequence aliment of VH chains of selected 11 clones

Group	clones	FWR1	CDR1	FWR2	CDR2
1	1E8, 1F6, 1H7	QVQLQQSGAEVKKPGASVKVSCKASGYNFA	S-HNMH	WVRQAPGQGLEWMG	IMK--PDNGRSRQTQKLRG
2	1E6	QVQLVESGSELKKPGASVKVSCTASGYTFN	N-YAMN	WVRQAPGQGLEWMG	YIN--TNTGKSTYAQGFTG
	1F4	....QQ.....	.-....	.....	.....
3	1E10	QVQLQQWGAGLLKPSETLSLTCAVSGGSLN	-GYYWN	WIRQPPGKGLEWIG	EIN---HSGSTNYNPSLKS
	1F1	....VES.G.VVQ.GRS.R.S..A..FT..	N-.GMQ	.V..A.....VA	L.S--HAGSDKY.GD.VKG
	1F9	......S..EVKR.GSSVKVS.KA...TFS	ST.AIS	.V..A..Q....M.	G.I--PR..TAKSAQN.QG
4	1G11, 1H11	QVQLQESGPGLVKPSETLSLTCAVSDYSVS	SDYFWG	WIRQSPGRGLEWIG	SIY---HSGSTYYDPSLKS
Other	1F5	QVQLLETGGGLVKPGGSLRLSCAASGFTFI	D-AWLS	WVRQAPGKGLEYIG	RIKKKKDGGTTDYAAPVKG

clones	FWR3	CDR3	FWR4
1E8, 1F6, 1H7	RLTLTRDTSTRTHYMELRNLKSEDTAIYYCAR	DSK----------FFGS--DY	WGQGTLVTVSS-------
1E6	RYVLSLDTSASTAYLQISSLKAEDTAVYYCAR	DSAG--------THRDL--DF	WGQGTLVTVSSGSASAPT
1F4	.....	....---------.....-..	..........-------
1E10	RVTMSVDTSRNQFYLKLDSVTAADTAVYYCAR	---G--------YYKR---DY	WGQGTLVTVSS-------
1F1	.F.I.R.N.KNTV..QMS.LR.D.....	DVVR--------SGDD----L	..........-------
1F9	...ITA.E.TSTV.ME.S.LRSD.....	DPG.TGAVNSYY..YGM--.V	..........-------
1G11, 1H11	RITISMDTSQNQFSLELRSLTAADTAVYFCAR	EKPGWHYYDSGTYYSSGAIDS	WGQGTLVTVSS-------
1F5	RFTISRDDSKSTLYLQMNSLKTEDTALYYCTT	DLLG--------GHDWN--CK	WGQGTLVTVSS-------

Table 2B. Deduced amino acid sequence aliment of VL chains of selected 11 clones

Group	clones	FWR1	CDR1	FWR2	CDR2
1	1E8, 1F6, 1H7	QSVLIQPP-SVSGSPGQSITISC	TGIGSDVWKLNL---VS	WYRQYPGKAPKLLIY	QGRRRPS
2	1E6	DIVLTQTPDSLAVSLGETATINC	KSSQSLLYDSNRRQYLA	WYQQKPGQSPKLLIY	WASTREA
	1F4	QPG.....-.VS.AP.K..S.T.	E--GNNIGSK.----VH	........A.L.V..	YD.DRPS
3	1E10	DIVLTQTPGTLSLSPGERATLSC	RASQSVGN-----TYLA	WYHQKPGQAPRLIIY	GASTRAT
	1F1	E.....S.....	......TG-----N...	..Q.....	...S...
	1F9	E.....S.....	......SS-----R...	..Q.....	...S...
4	1G11, 1H11	SYELMQPP-SLSVSPGQTARI--	----------	----------	-------
Other	1F5	DIQVTQSPSSLSASVGDTVTITC	RASQSVGD------WVA	WYQLKPEGGPKSLVF	ASSAVQN

clones	FWR3	CDR3	FWR4
1E8, 1F6, 1H7	GISDRFSGSKSGNTASLTISGLQAEDEADYYC	SSYRS--SSTWV	FGGGTKLTVL
1E6	GVPDRFSGSVSGTDFTLTINSLQAEDVAVYYC	LQYYR----SLN	FGPGTKVDIK
1F4	...K.....N..NTA....SRVE.G.E.D...	QVWQSNIDHPRV	..G...LTVL
1E10	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGS---SQYT	FGQGTKVEIK
1F1	.............V.....	.....---.PW.	.....
1F9	.....	....NP--.TW.	.....
1G11, 1H11	----------	----------T	FGQGTRLEIK
1F5	GVPSRFSGRGYGTDFTLTISDLQPEDSATYYC	QQYDD---LPIT	FGQGTRLEIK

Table 3. Eleven clones with affinity for T-antigen are categorized into 4 groups based on their amino acid sequences

Group	Clone names	Primary structure of scFvs (Table 2)
1	1E8, 1F6, 1H7	Three clones are identical.
2	1E6, 1F4	VH domains are nearly identical while VL domains differ.
3	1E10, 1F1, 1F9	VL domains are nearly identical while VH domains differ.
4	1G11, 1H11	Two clones are identical, but lack most of the VL domain.
NA	1F5	This clone is different from others although significant homology to CDRs of other clones is present.

Clones underlined, 1E8, 1E6, 1E10, and 1G11, were used for further studies.

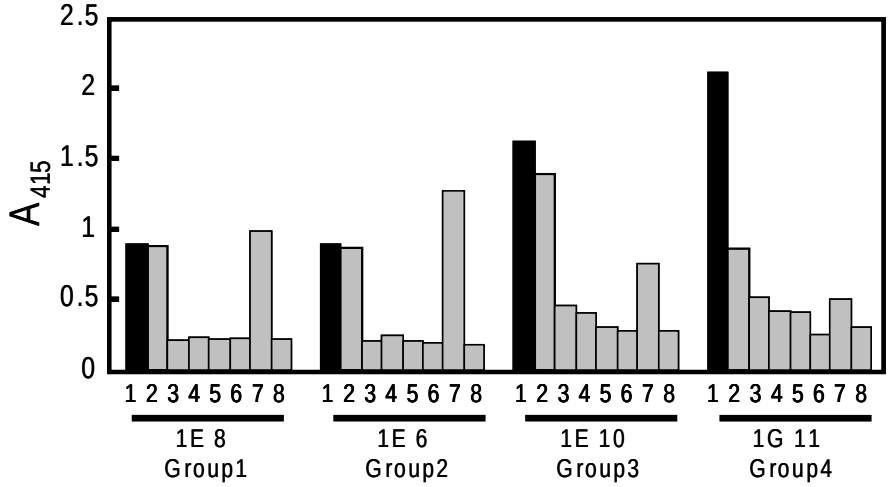

Figure 2. Binding activities of 4 phage antibodies, 1E8, 1E6, 1E10, and 1G11 representing Group 1, 2, 3, and 4 (Table 2), respectively. Various neoglycolipids in addition to T antigen-E6-BDB were immobilized in wells of a 96-well plate. Shown are binding of each phage antibody to T-antigen-E6-BDB (1: ■), Tn-antigen-E6-BDB (2), LnT- (3), LnNT- (4), LNFPI- (5), LNFPII- (6), LNFPIII- (7), and LNDFHI-DPPE (8).

cultured in the presence of IPTG to induce scFv protein expression. Cells collected were solubilized with a non-ionic detergent, from which the1G11 scFv protein was purified by DEAE-cellulose and Ni^{2+}-Sepharose affinity chromatographies. The scFv protein was eluted from a DEAE-cellulose column by a 0-1 M NaCl gradient. Eluted fractions were analyzed by SDS-PAGE followed by CBB staining and Western blotting with anti-E-tag antibody. As shown in Figure 3A and 3B, respectively, the results indicated that fractions 21 to 26 contained the anti-E-tag detectable 1G11-scFv protein. Fractions 21 to 26 were combined and then subjected to SPR analyses using T-antigen conjugated human serum albumin (T-antigen HSA) (Figure 4A). The combined DEAE-cellulose fractions revealed higher binding activity to T-antigen HSA than the crude cell lysates which had been applied to the DEAE-cellulose column (sensorgrams a and b in Figure 4A, respectively). The 1G11 scFv fractions eluted from the DEAE-cellulose column were subsequently purified by Ni^{2+}-Sepharose affinity chromatography. Under non-denaturing conditions, however, 1G11 scFv did not bind to Ni^{2+}-Sepharose gel (data not shown), which suggested that the His-tag located at the C-terminus of 1G11 scFv may not be accessible to Ni^{2+} due to possible steric hindrance. Alternatively, 1G11 scFv was purified by Ni^{2+}-Sepharose chromatography in the presence of 2 M urea. The 1G11 scFv protein was purified to near homogeneity as revealed by SDS-PAGE/CBB staining (Figure 3C) and Western blotting with anti-E-tag antibody (Figure 3D). The yield of the purified 1G11 protein was 85 μg from 3.56 mg (wet weight) of *E. coli* or 312 mg protein of the solubilized fractions.

3.4. *Binding activities of the purified 1G11 scFv protein*

The binding activity of the purified 1G11 scFv protein was determined by ELISA. As shown in Figure 4B, ELISA was carried out with serial dilutions of the purified 1G11-scFv using T-antigen E6-BDB, Tn-antigen E6-BDB, E6-BDB, and BSA. The results indicated that the purified 1G11 scFv has higher binding activity for T-antigen than Tn-antigen.

4. Discussion

In this manuscript, isolation and characterization of anti-T-antigen scFvs were described. Of 96 phage clones screened from the phage library, 11 clones were shown to carry scFv sequences and had affinity for T-antigen E6-BDB, a carbohydrate probe presenting T-antigen. Of note is that a significantly high ratio of positive clones, 11.5% of clones screened, was obtained. When anti-M3 scFvs were previously isolated using M3-

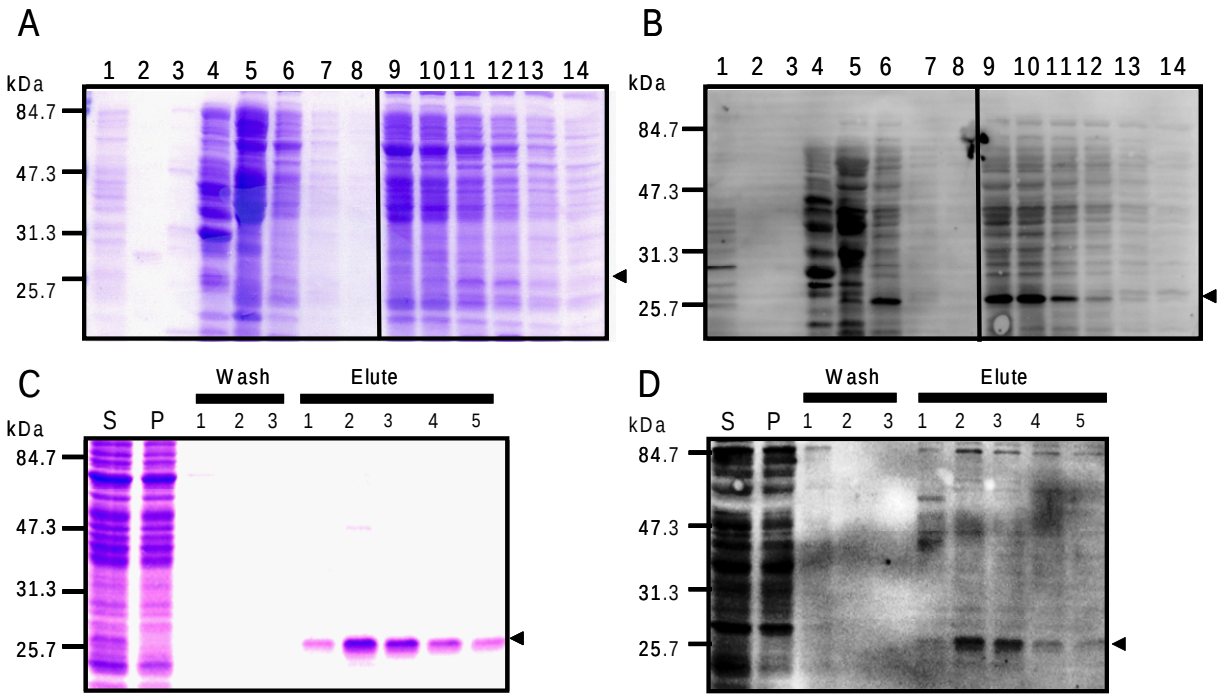

Figure 3. Analyses of the 1G11 scFv protein fractions eluted from DEAE-cellulose (A and B) and Ni^{2+}-Sepharose (C and D). In A and B, SDS-PAGE and Western blotting, respectively, of DEAE-cellulose eluates are shown: the solubilized fraction (lane 1), the passed-through fraction (lane 2), DEAE-cellulose eluate combined fractions #1-10 (lane 3), fractions #11-16 (lane 4), fractions #17-20 (lane 5), fractions #21-26 (lane 6), fractions #27-34 (lane 7), and fractions #35-42 (lane 8). Lanes 9-14 show each fraction of #21-26, respectively, which were subjected to Ni^{2+}-Sepharose affinity chromatography. In C and D, SDS-PAGE and Western blotting, respectively, of Ni^{2+}-Sepharose eluates are shown: DEAE cellulose fractions #21-26 (S), the passed-through fractions (P), fractions #1-3 washed with PBS containing 40 mM imidazole (Wash: lanes 1-3), fractions #1-5 eluted by PBS containing 400 mM imidazole (Elute: lane 1-5). Protein bands were stained with CBB (A and C). Western blotting with anti His-tagged mouse monoclonal antibody followed by anti-mouse IgG-HRP was carried out to stain the 1G11 scFv protein (B and D).

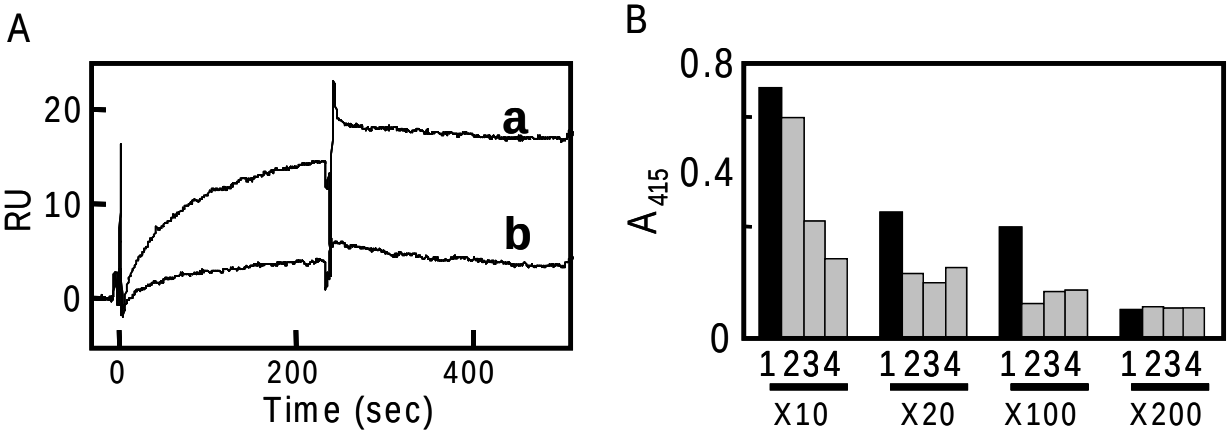

Figure 4. Tn-antigen binding activity of the purified 1G11 scFv protein. A: SPR analysis of the DEAE-cellulose eluates (a) and solubilized fractions (b). Binding to T-antigen HSA is shown. B: ELISA was carried out to determine T-antigen binding activity of the affinity purified 1G11 scFv (42.5 μg/mL). Shown are dose dependencies of binding activities against T-antigen E6-BDB (1: ■), Tn-antigen E6 BDB (GalNAcα-) (2), E6-BDB (3) and BSA (4).

DPPE as an antigen (*14*), only 15 out of 672 clones (2.2%) with intact scFvs were obtained. In addition, unlike isolation of M3 scFvs in which 15 clones had all different sequences, three and two identical clones were obtained for Group 1 and 4 phages, respectively, when screened by T-antigen E6-BDB. Such a concentration of binding to an antigen during panning is a good indication of possible positive clones being screened. The different outcomes from two studies may be due to the nature of their different carbohydrate moieties or the difference in the design of neoglycolipids used, namely the presence of E6 in the case of screening T-antigen scFvs. In contrast, panning and screening of phages with Tn-antigen E6-BDB did not yield any positive

clones although 928 clones were screened by ELISA. These results clearly suggested that while the binding of T-antigen consisting of disaccharide to antibodies can be achieved, Tn-antigen, which is the monosaccharide, is not big enough to stably bind to the binding domains of antibodies.

Characterization of phages isolated with T-antigen E6-BDB revealed that there are largely four different scFvs, Group 1-4. Group 4 consisting of two identical clones, 1G11 and 1H11, apparently showed high affinity and specificity for T-antigen when analyzed as a phage antibody, but are incomplete forms of scFvs, consisting of VH domain and only 30 amino acids of VL domain consisting of FWR1 and FWR4 and lacking most of the VL domain, CDR1, FWR2, CDR2, FWR3, and CDR3. Although it was obtained as an artifact in this case, since these clones were derived from a phage library with a large repertoire of natural and engineered human scFv genes, it resembles heavy-chain antibodies, so-called camelids (*19,20*). Camelids possess a functional class of antibodies devoid of light chains. The Group 4 clones may provide us with experimental models to test the role of VL domains in the formation of antigen-binding sites.

Besides binding to T-antigen, those phage antibodies except the Group 4 clone showed crossreactivity to Tn-antigen and LNFPIII. It is ultimately required to express and purify scFv proteins before their binding affinities and specificities can be evaluated. As a first step, 1G11 scFv (Group 4) protein was purified to near homogeneity by two column chromatographies from soluble fractions of *E. coli* expressing the scFv protein. The purified 1G11 scFv bound to T-antigen with greater affinity than Tn-antigen, but its binding activity seemed to be much lower than expected. This was also supported by SPR analyses which did not yield sensorgrams comparable to that of DEAE-cellulose eluates (Figure 4A-a). These results indicated that the purified 1G11 may be inactivated as a result of exposure to 2 M urea during Ni^{2+}-Sepharose affinity chromatography and that refolding had not been completed during elution with 400 mM imidazole/PBS. It is obvious that we need to improve procedures for expression and purification of the fractions for further characterization of scFv proteins and determination of affinity constants. Expression of Group 1-4 scFv proteins in *E. coli* at high levels and subsequent purification are now in progress, and in fact, a binding constant (KD) of 8×10^{-7} M for T-antigen HSA has been obtained for one of the scFv proteins purified (manuscript in preparation). Such production and purification of anti-T-antigen scFv proteins would provide the basis for developing antibody-based cancer therapeutics.

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