J. Biochem. 2010;147(6):809–817 doi:10.1093/jb/mvq014

JB THE JOURNAL OF BIOCHEMISTRY

Isolation and characterization of antibodies against three consecutive Tn-antigen clusters from a phage library displaying human single-chain variable fragments

Received December 7, 2009; accepted January 25, 2010; published online February 10, 2010

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The Tn-antigen, GalNAcα-Ser/Thr, is a tumour-associated carbohydrate antigen that may provide a sensitive and specific marker for pre-clinical detection of carcinoma and a target for cancer therapies. We recently reported that MLS128 monoclonal antibody treatment significantly inhibited colon and breast cancer cell growth. On the basis of our observations, the present study aimed to produce human anti-Tn-antigen antibodies with specificity similar to that of MLS128 monoclonal antibody, which recognizes a structure of three consecutive Tn-antigens (Tn3). Six phage clones displaying human single-chain variable fragments (scFvs) were isolated from a newly constructed phage library by panning and screening with a synthetic Tn3-peptide. Deduced amino-acid sequences of six anti-Tn3 scFvs exhibited a high degree of homology. Of those, anti-Tn3 4E10 and 4G2 scFv proteins were successfully purified from phage-infected *Escherichia coli* to near homogeneity. Surface plasmon resonance analyses revealed a K_D of purified scFv proteins for Tn3 on an order of 10^{-7} M, which is high for carbohydrate-specific monovalent antibodies. Further analyses suggested that both scFv proteins also bind to Tn2 and cultured colon and breast cancer cells. These results demonstrated the potential for use of these scFvs in developing antibody therapeutics targeting colon and breast cancer.

Keywords: Tn-antigen/single chain Fv/phage display/human antibody/colon and breast cancer cells.

Abbreviations: Ab, antibody; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; BSM, bovine salivary gland mucin; CDR, complementarity determining region; CBB, Coomassie Brilliant Blue; cfu, colony formation unit; DIEA, *N*,*N*-diisopropylethylamine; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fmoc, (9H-fluoren-9-ylmethoxy)carbonyl; FWR, framework region; Fv, variable fragment; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; mAb, monoclonal Ab; MEM, Minimum Essential Medium; NMP, *N*-methylpyrrolidone; PyBOP, (benzotriazol-1-yloxy) tripyrrolidinophosphonium·hexafluorophosphate; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; scFv, single chain variable fragment; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; Tn3, three consecutive Tn-antigens (GalNAcα-Ser/Thr); VH, variable region of antibody heavy chain; VL, variable region of antibody light chain.

Oncogenic transformation is often associated with dysregulation of glycosylation processes, which leads to altered patterns of carbohydrate constituents on the surface of cancer cells. Some of these tumour-associated carbohydrate antigens are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. The Tn-antigen, GalNAcα-Ser/Thr, is one such antigen associated with carcinomas, which provides a sensitive and specific marker for pre-clinical detection of carcinoma and a specific therapeutic target for cancer treatment (*1–4*).

The present study sought to produce human anti-Tn-antigen antibodies with specificity similar to that of MLS128 monoclonal antibody (mAb). This mAb was derived from a mouse immunized with LS180 human colon carcinoma cells and determined to be IgG_3 (*5*). MLS128 recognizes a structure of three consecutive Tn-antigens (Tn3) (*6*, *7*). The current authors recently reported the following findings (*8*): (i) MLS128 treatment significantly inhibited colon and breast cancer cell growth, (ii) MLS128 bound to 110–210 kDa glycoproteins on the cell surface and (iii) MLS128 treatment caused down-regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor in LS180 cells, suggesting that

MLS128-inhibited cancer cell growth is in part mediated by down-regulation of growth factor receptors. Based on these findings, human-type antibodies against Tn3, which is the epitope for MLS128, were screened from a phage library displaying human single-chain variable fragments (scFvs). This article describes isolation of phage antibodies and purification and characterization of human-type scFv proteins with a high level of affinity for Tn3 that can be readily used to develop potential cancer therapeutics.

Materials and Methods

Materials

Helper phage M13KO7, horseradish peroxidase (HRP)-labelled anti-M13, Ni^{2+}-Sepharose and isopropyl β-D-thiogalactoside (IPTG) were purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). BugBuster, EzWest Blue and His-probe-HRP were purchased from Novagene Merck (Darmstadt, Germany), ATTO (Tokyo, Japan) and Pierce (Rockford, IL, USA), respectively. Covalink plates were from Nunc (Roskilde, Denmark). Human IgA_1 was kindly provided by Dr Hitoo Iwase (Kitasato University, Kanagawa, Japan) (*9*). MLS128 was provided by Dr Hiroshi Nakada (Kyoto Sangyo University, Kyoto, Japan). Human colon adenocarcinoma LS180 and human breast carcinoma MCF-7 cells were obtained from American Type Culture Collection. MAT-tag antibody from murine myeloma, anhydrotetracycline and spectinomycin were purchased from Sigma-Aldrich (St Louis, MO, USA). 1-Arabinose and carbenicillin were from Wako Pure Chemical (Osaka, Japan). Cy3-conjugated AffiPure goat anti-mouse IgG was from Jackson Immunoresearch Inc. (West Grove, PA, USA). *Escerichia coli* strains, the suppressor strain XL1-Blue and the nonsupressor strain TOP10F′ were obtained from Invitrogen (Carlsbad, CA, USA). *Escherichia coli* XL1-blue FS2 [XL1-blue in which periplasmic chaperon vector (pFS) containing *fkpA* and *skp* (*10*, *11*) was incorporated] was used for expression of phage-displaying scFv, while *E. coli* Top10F′ DFS [TOP10F′ in which *dsbA* containing periplasmic chaperon was incorporated] was used for expression of scFv proteins. Reagents for syntheses of Tn-peptides were commercially obtained and used without further purification. All other solvents were purchased from Kanto Chemical Co. (Tokyo, Japan) as dry solvents.

Cell culture

LS180 cells were cultured in MEM containing 10% fetal bovine serum (FBS), 50 U/ml of penicillin–streptomycin and 0.2% Fungizone (Invitrogen). MCF-7 cells were cultured in Improved MEM containing 5% FBS and 11.25 nM insulin (Sigma-Aldrich).

Synthesis of Tn3-peptides

The glycopeptides containing three Tn-antigens, glycopeptides 3 and 5 as shown in Fig.1 and Table I, were synthesized as follows. Solid-phase synthesis of glycopeptide 3, the Tn3-peptide with a free *N*-terminus, was carried out starting with commercially available Fmoc-Gly-Wang resin (0.136 g, 0.0816 mmol), to which Fmoc-ß-Ala-OH, Fmoc-Thr[GalNAc(OAc)$_3$]-OH and Fmoc-Gly-OH were sequentially coupled (three times for Fmoc-Thr[GalNAc(OAc)$_3$]-OH) (*12*). Every coupling cycle consisted of the cleavage of the *N*-terminal Fmoc-protecting group, followed by coupling of the subsequent Fmoc-amino acid. Cleavage of the Fmoc group was achieved by treatment of the resin with a solution of piperidine (20%) in *N*-methylpyrrolidone (NMP) for 3 min twice. A mixture of (Benzotriazol-1-yloxy) tripyrrolidinophosphonium·hexafluorophosphate (PyBOP) (5 eq.) and *N*,*N*-diisopropylethylamine (DIEA) (10 eq.) in NMP was used for coupling of the Fmoc-amino acids (5 eq.). The coupling time was 40–60 min. The Tn-Thr building block (3 eq.) was dissolved in NMP and coupled with PyBOP (3 eq.) and DIEA (6 eq.). Coupling reactions were confirmed by the Kaiser test (*13*). After coupling of all amino acids, Fmoc groups were removed with piperidine (20%) in NMP. Cleavage of the resin was carried out in 95% aq. TFA to give glycopeptide 2. All *O*-acetyl groups of compound 1 were removed with (*n*-Bu_4N)OH (1.0 M) in H_2O. The crude product was purified by a preparative RP–HPLC with a gradient of 70–50% CH_3CN in water (0.1% TFA) over 60 min using a TOSOH TSKgel Amide-80 column (10 μm, 21.5 × 300 mm, 10 ml/min) to give glycopeptide 3 (15.9 mg, 0.0142 mmol, 18%). MALDI-TOF MS (α-CHCA, positive): *m*/*z* calcd for $C_{43}H_{73}N_9NaO_{25}$: 1138.5; found: 1139.1 ($M + Na^+$). Preparative RP-HPLC separations were performed on a Hitachi L-6250 HPLC system and Hitachi L-4000 UV detector. MALDI-TOF mass spectra were recorded on a Voyager-DE (Applied Biosystems, Foster City, CA, USA). Similarly, an N-terminus capped Tn3 peptide, glycopeptide 5, was synthesized from Fmoc-Gly-Wang resin (0.085 g, 0.051 mmol). Introduction of an acetyl group to an amino group of glycopeptide 2 was carried out with excess Ac_2O and DIEA in NMP. Cleavage of the resin was carried out using 95% aq. TFA. All *O*-acetyl groups were removed with (*n*-Bu_4N)OH (1.0 M) in H_2O. The crude product was purified by preparative RP-HPLC with a gradient of 75–50% CH_3CN in water (1% TFA) over 60 min to give glycopeptide 5 (9.5 mg, 0.0082 mmol, 16%). MALDI-TOF MS (2,5-DHB, positive): *m*/*z*

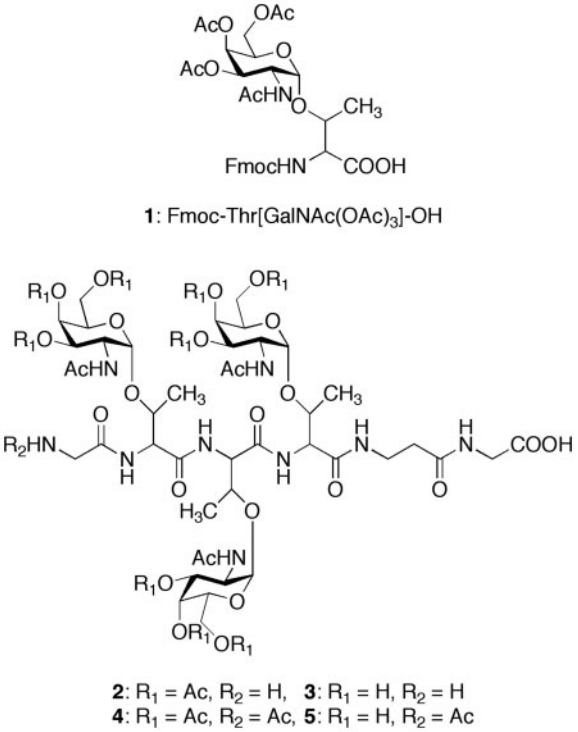

Fig. 1 Structures of glycopeptides with three consecutive Tn-antigen clusters (Tn3-peptides). Tn3-peptides were synthesized as described in 'Materials and Methods' section. Shown are glycopeptides **1**–**6** with Fmoc for **1** or R_1 and R_2 as indicated for **2**–**6**.

Table I. List and structures of glycopeptides used in this study.

Tn peptides	Structure
Tn0	Acetyl-Gly-Thr-Thr-Thr-βAla-Gly-OH Gly-Thr-Thr-Thr-βAla-Gly-OH
Tn2	Acetyl-Gly-Thr-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH Gly-Thr-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH
Tn3	Acetyl-Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH: Glycopeptide 3 (Fig.1) Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH: Glycopeptide 5 (Fig.1)

calcd for $C_{45}H_{75}N_9NaO_{25}$: 1180.47; found: 1180.8 ($M + Na^+$), calcd for $C_{45}H_{75}KN_9O_{25}$ 1196.4; found: 1197.1 ($M + K^+$).

Construction of a phage library displaying scFv antibodies

A new scFv-displaying phage library was constructed as similar to a previously reported phage library (*14*). The changes in the new library were replacement of the tag sequences with MAT tag (Sigma-Aldrich) sequence and insertion of a trypsin-cleavable sequence between svFv and g3 protein. Details will be published elsewhere (manuscript in preparation). Procedures for construction of the VH and VL sub-libraries with $>10^7$ colonies were similar to those previously reported (*12*). The repertory of scFv genes resulting from the recombination of sub-libraries was calculated to be $>10^{11}$.

Panning and screening of phages displaying human scFvs against Tn3

The phage library was subjected to three rounds of affinity selection using N-terminal blocked Tn3 peptide (glycopeptide 3 in Fig. 1 and Table I). Eight wells, four wells and two wells were prepared as follows for the first, second and third panning, respectively. Tn3 peptides were covalently linked to the free amino groups on Covalink 96-well plates *via* carboxyl groups using water-soluble carbodiimide, EDC, as coupling reagent. Briefly, 25 µl of 50 µM Tn3 peptide and 2.5 mM EDC in water were added to Covalink wells and allowed to react for 2 h at 37°C. After blocking with 3% BSA/TBS (10 mM Tris–HCl, pH 7.4 containing 150 mM NaCl) overnight at 4°C, 50 µl of the phage library solution (10^{12} cfu/ml) were added to each well in TBS containing 0.1% Tween-20 and 3% BSA and left to incubate for 2 h at 37°C. The wells were washed with TBS containing 0.1% Tween-20 and TBS; five times for the first round, 10 times for the second round and 20 times for the third round. After panning, phages bound to the antigen were recovered by incubation with 50 µl of TrypLE Express (Invitrogen) for 5 min at room temperature. Phage particles amplified with *E. coli* XL1-Blue FS2 and a helper phage were concentrated by PEG-precipitation and dissolved in 700 µl of TBS, 700 µl of 3% BSA/TBS and 1 µl (25 units) of Benzonase (Novagen) by incubation at 37°C for 1 h. After centrifugation at 18,000*g* for 5 min at 4°C, phage suspensions were recovered and used for subsequent panning. Subtraction panning was done two and four times during the second and third panning, respectively. After three rounds of panning, phages derived from 192 single colonies were subjected to screening by ELISA using the Tn3 peptide as an antigen.

Characterization of phage antibodies

scFv genes were amplified from phagemids prepared from *E. coli* XL1-Blue FS2 infected with phages by PCR with a primer set (forward primer: 5′-ATGGCTTCCATGTCGGCAGA-3′, reverse primer: 5′-GCCAGCATTGACAGGCTAG-3′) as previously described (*14*). After purification by 2% agarose gel electrophoresis, the scFv genes were subjected to DNA sequencing (ABI PRISM Model 3100, Applied Biosystems).

Production of scFv proteins

Each phage-infected TOP10F′ DFS was grown overnight in a test tube at 30°C with shaking at 250 rpm in SBS (LB-10 mM Tris–HCl, pH 7.5) containing 50 µg/ml carbenicillin and 10 µg/ml spectinomycin. The culture was continued in a flask containing SBS media, 0.4% glycerol, 50 µg/ml carbenicillin and 10 µg/ml spectinomycin. After culturing at 25°C for 2 h at 250 rpm, expression of scFv protein was induced by adding 10 µg/ml anhydrotetracycline and 0.01% L-arabinose and culturing the result overnight at 25°C.

Preparation and purification of scFv proteins

To prepare periplasmic extracts, cells collected from a 50-ml culture were suspended in 1 ml of 0.2 M Tris–HCl, pH 8.0, containing 0.5 mM EDTA and 0.5 M sucrose (TES) and this was followed by adding 1.5 ml of ice-cold 5-fold diluted TES and incubating the cells on ice for 15–30 min. The pellet was removed by centrifugation at 10,000*g* for 40 min at 4°C. The supernatants were recovered as periplasmic extracts. The pellet was suspended in 2 ml of non-ionic detergent BugBuster (Novagen) containing a protease inhibitor cocktail (Sigma-Aldrich; P8340) and lysozyme (Novagen) and then rotated for 1 h at room temperature. After centrifugation at 10,000*g* for 50 min at 4°C, supernatants were recovered as cytosolic extracts. The periplasmic extracts were treated with 0.1% NP-40. Soluble scFv proteins from the periplasmic and cytoplasmic extracts were purified by Ni^{2+}–Sepharose affinity chromatography. Briefly, 100 µl of Ni^{2+}–Sepharose were added to the extracts and allowed to bind scFv proteins by rotating overnight at 4°C. The Ni^{2+}–Sepharose was packed in a column. After washing thoroughly with 100 mM sodium phosphate buffer, pH 7.4, containing 20 mM imidazole and 0.1% NP-40, bound scFv proteins were eluted with 100 mM sodium phosphate buffer, pH 7.4, containing 0.4 M imidazole, 0.5 M NaCl and 0.1% NP-40. The eluted scFv proteins were dialysed against TBS. Protein amounts were determined by a BCA protein assay kit (Pierce).

SDS–PAGE and western blotting

The purified scFv proteins (0.5 µg/lane) were analysed by SDS–PAGE (4–20% acrylamide gel) (ATTO) under reducing conditions. For immunoblotting, the proteins on the SDS–PAGE gel were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with PBS containing 3% BSA for 2 h at 37°C. After it was washed with PBS, the membrane was incubated with HRP-conjugated His-probe or anti-MAT antibody at room temperature for 1 h. The membranes were washed with PBS five times. For anti-MAT antibody immunoblotting, the membrane was incubated with HRP-conjugated anti-mouse IgG Ab for 1 h at room temperature. After both membranes were washed with PBS containing 0.2% Triton X-100 and PBS five times each, scFv proteins were visualized by colour development using EzWestBlue (ATTO).

Determination of affinity and specificity

Real-time measurement of the binding of 4E10 and 4B2 scFv proteins to the Tn3-peptide was performed by surface plasmon resonance (SPR). All SPR analyses were carried out at 25°C on a Biacore 3000 biosensor (GE Healthcare, Uppsala, Sweden). N-terminus free Tn3- and Tn0-peptides (Table I) were immobilized on CM3 sensor chips using the amine coupling kit supplied by the manufacturer. Binding of scFv proteins to carbohydrate moieties immobilized on the surface of the sensor chips was monitored at a flow rate of 20 µl/min. The rates of association (*k*a) and dissociation (*k*d) for 4E10 and 4G2 scFv proteins were determined by a monovalent analyte model using BIAevaluation version3.0 software. The dissociation constant (K_D) was derived by dividing *k*d by *k*a.

ELISA was used to evaluate the antigen-binding activity of purified 4E10 and 4G2 scFv proteins. Tn3, Tn2 and Tn0 peptides were coated as described above. An amino plate (ImmobilizerTM-Amino Modules; Nunc) was used to covalently link Tn-peptides, whereas a Medisorp plate (Nunc) was used to coat IgA_1. Fifty microlitres of IgA_1 (1 µg/ml) in PBS was applied to wells on a 96-well-plate. The plate was incubated at room temperature overnight, blocked with 3% BSA/TBS for 2 h at 37°C and rinsed with TBST. To antigen-coated wells, 50 µl of scFv proteins (30 µg/ml) or MLS128 (3 µg/ml) were added and allowed to bind to antigens for 2 h at 37°C. After they were washed five times with TBST, the wells were incubated with 1 µg/ml of HRP-labelled His-probe and anti-mouse IgG Ab (DAKO, Glostrup, Denmark) for scFv proteins and MLS128 mAb, respectively, for 1 h at room temperature. The wells were washed five times with TBST and then 10 times with TBS. The bound scFv proteins or mAb were detected by colour development using a 100 µl ABTS solution (Roche Diagnostics, Basel, Switzerland), and the reaction was stopped by adding 100 µl of 2% oxalic acid. Absorbance at 415 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA).

Immunofluorescence microscopy

Colon cancer LS180 and breast cancer MCF-7 cells were grown on collagen-coated 10-well glass slides (Matsumi Glass, Osaka, Japan) by seeding 50 µl of freshly prepared cells (suspended in culture medium: 5×10^5 cells/ml) and culturing them for 24 h. Attached cells were fixed with a Zamboni solution (phosphate-buffered 4% paraformaldehyde with picric acid) for 15 min. Cell-coated wells were quickly washed with PBS and incubated for 3 h at 37°C with scFv proteins or MLS128 mAb 50 µg/ml. After they were washed five times with PBS, cell-coated wells were incubated with anti-MAT-tag antibody for 1 h at room temperature. The cell-coated

wells were washed with PBS, and then bound antibodies were detected by binding to Cy3-conjugated anti-mouse IgG Ab. Slides were washed three times with PBS, mounted with PBS/30% glycerol containing DABCO solution (Wako), and then examined with Keyence fluorescence microscopy (BZ9000; Keyence, Osaka, Japan).

Results

Screening of scFv-displaying phages directed against Tn3-peptide

A phage displayed human scFv library with the phagemid vector construct was subjected to three rounds of panning against Tn3 peptide, glycopeptide 3, with a structure as shown in Fig. 1 and Table I. Of 192 colonies screened by ELISA using Tn3/Tn0 peptides (Table I), 13 positive clones were isolated. From those, six clones with a Tn3/Tn0-peptide ratio of >1.7 (Table II) were selected for further analyses. DNA sequencing of scFv regions of the selected phage clones revealed that all of the clones differed. Deduced amino-acid sequence alignments of VH and VL are presented in Tables III and IV, respectively. With the exception of 4A7, five clones share extensive homologies in VH CDR1 and 2. Significant homologies are found in VL CDR1 and two among five clones except for 1A11. In contrast, clones 4A7 and 4G10 lack VH CDR3, which is found to vary the most among the four other clones (Table III). Clones 4E10 and 4G2 were selected for expression of scFv genes with the same amino-acid sequences in the regions of VH CDR1 and 2 but with differences to a certain extent in the regions of VH CDR3 and VL CDR1/2/3.

Table II. Six scFv-displaying phages isolated from a new phage library by panning and screening with Tn3-peptide.

Clones	Binding to Tn3 peptide (Tn3 /T0 peptide ratio)
1A11	1.7
4A7	1.8
4E10	2.2
4F12	2.0
4G2	1.9
4G10	2.2

Expression and purification of scFv proteins

4E10 and 4G2 proteins expressed in *E. coli* TOP10F′ DFS infected with 4E10 or 4G2 phages were purified by Ni^{2+}–Sepharose from both periplasmic and cytosolic extracts. The amounts of the scFv proteins purified from periplasm and cytosolic extracts from a 100-ml culture were 50 and 275 μg, respectively, for 4E10, and 32.5 and 280 μg, respectively, for 4G2. Passed-through (PT) and eluted (E) fractions prepared from periplasmic extracts were analysed by SDS–PAGE and immunoblotting (Fig. 2). The CBB-stained SDS–PAGE gel revealed purified 4E10 and 4G2 scFv proteins with a molecular mass of ~28 kDa (Fig. 2A) that were then immunostained with either His-probe (Fig. 2B) or anti- MAT antibody (Fig. 2C). These results indicated both 4E10 and 4G2 scFv proteins were purified from periplasmic extracts to near homogeneity by Ni^{2+}–Sepharose chromatography. In contrast, the scFv proteins purified from the cytosolic extracts were estimated to be ~85% pure (data not shown).

To compare binding activities of these scFv proteins purified from periplasmic and cytoplasmic extracts, ELISA was performed using Tn3-peptide as an antigen and Tn0-peptide as a control. 4E10 and 4G2 scFv proteins from periplasmic extracts had similar specific activity (Fig. 3). Both periplasmic-derived scFv proteins had higher specific activities than those of cytoplasmic-derived scFv proteins. It is likely that the lower specific activities observed with cytoplasm-derived scFvs are at least in part due to immature refolding and lower purity.

Table III. Deduced amino-acid sequence alignment of VH chains of six selected clones.

	FWR1	CDR1	FWR2
1A11	QVQLQQSGPGQVKPSQSLSLTCAISGDSVS	SNRAAWN	WIRQSPSRGLEWLG
4A7	QVQLQESGPGLVKPSETLSLTCTVSDGSIS	NYYWN	WIRQPPGKGLEWIG
4E10	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG
4F12	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSASWN	WIRQSPSRGLEWLG
4G2	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG
4G10	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG

	CDR2	FWR3
1A11	RTYYRSQWYHDYAESLKS	RITITPDSSKNQFSLQLNSLTPEDTAMYYCAR
4A7	SFAYSFAY	
4E10	RTYYRSKWYNDYAVSVKS	RITINPDTSKNHFSLHLNSVTPEDTAMYYCTT
4F12	RTYYRSKWYYDFATSVKS	RIVIQPDTSKNQFSLQVNSVTPEDTAMYYCAR
4G2	RTYYRSKWYNDYAVSVKS	RITINPDTSKNQFSLQLYSVTPEDTAMYYCVR
4G10	RTYYRSKWYNDYAVSVNS	RITINPDTSKNQFSLQLNSVTPEDTAMYYCAP

	CDR3	FWR4
1A11	SPQGYDFWSGYYGDS	WGQGTLVTVSS
4A7		
4E10	DQLFNDH	WGQGTLVTVSS
4F12	GPYCGSDSCYERYYYGMDV	WGPGTTVTVSS
4G2	GNNTGWFPF YYGLDV	WGQGTTVTVSS
4G10		TLVTVSS

Table IV. Deduced amino-acid sequence alignment of VL chains of six selected clones.

	FRW1	CDR1	FRW2
1A11	QPGLTQPPSASALLGASIKNTC	TLGSEDSTHTIK	WNQQRPGRSPQYIMKLKTDG
4A7	QPGLTQPPSASGTPGQRVTIPC	SGSSSNIGGKTVN	WYQQLPGTAPKLLIY
4E10	NFMLTQPPSASGTPGQTVTISC	SGGSSNIGSWPVS	WYQQLPGTAPKLLIY
4F12	NFMLTQPPSASGTPGQRVTISC	SGSSSNTGSNTGN	WYQQLPGMAPKLLIY
4G2	NFMLTQPPSASGSPGQSVTISC	TGTSSDVGGYNFVS	WYQQHPGKAPKLIIY
4G10	NFMLTQPPSVSGTPGQRITISC	SGSNSNIGSDSVN	WYQHLPGAAPKLLMY

	CDR2	FRW3
1A11	SHIKGD	GIPDRFMGSSFGADRYLTFSNLQSDDEAEYHC
4A7	SNDHRPS	GVPDRFSASKSGTSASLTFSGLQSGDESDYYC
4E10	SNDQRPS	GVPDRFSGSKSGTSASLAISGLQSEDESDYYC
4F12	TNDQRPS	GVPDRFSGSKSGTSASLVISGLQSEDESDYYC
4G2	EVNKRPS	GVPDRFSGSKSGNTASLTVSGLQSEDESDYYC
4G10	TNRRRPS	GVPDRFSGSQSGTSASLAISGLQSEDESDYYC

	CDR3	FRW4
1A11	AAWDDSLKAWV	FGGGTKLTVL
4A7	NSRDSSGNHVV	FGGGTQLTVL
4E10	QSADISGTYAV	FGGGTQLTVL
4F12	ALYMGGGIWV	FGGRTKVTVL
4G2	VLYMGGGIYV	FGTGTKLTVL
4G10	AAWDDSLRTWV	FGGGTKLTVL

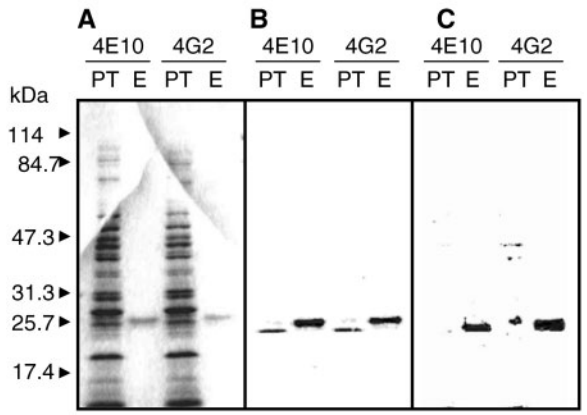

Fig. 2 SDS–PAGE and immunoblotting of purified 4E10 and 4G2 scFv proteins. (**A**) Coomassie Brilliant Blue (CBB)-staining on 4–20% acrylamide gel. (**B**) Immunoblotting with His-probe. (**C**) Immunoblotting with anti-MAT-tag antibody. PT and E are the passed-through fractions and 400 mM Imidazole-eluted fractions from a Ni^{2+}–Sepharose column, respectively.

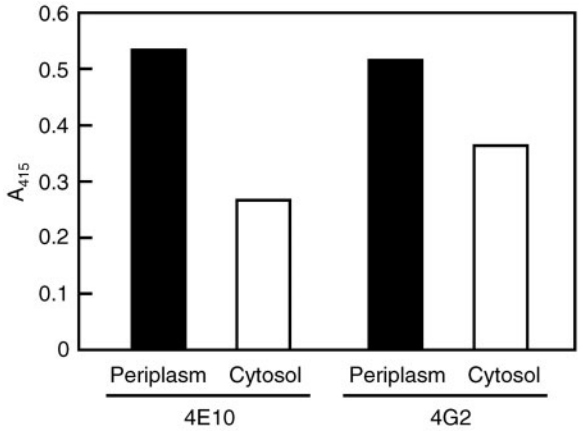

Fig. 3 Comparative binding activities of 4E10 and 4G2 scFv proteins purified from periplasmic and cytosolic extracts. ELISA was performed with 5 μg of purified proteins/well using Tn3-peptide or Tn0-peptide as described in 'Materials and Methods' section. Shown are Tn3 peptide binding activities of 4E10 and 4G2 scFv proteins purified from periplasmic (closed column) and cytoplasmic (open column) fractions.

SPR analyses of 4E10 and 4G2 scFv proteins against Tn3

SPR studies were performed to quantitatively measure scFv protein binding to Tn3-peptide. Sensorgrams showing the binding of 4E10 and 4G2 scFv proteins to Tn3-peptide immobilized on a sensor chip revealed their clear binding kinetics (Fig. 4A and B, respectively). The K_D was calculated on the basis of the measured *k*a and *k*d rate constants by a monovalent analyte model using BIAevaluation version3.0 software. Table V summarizes the kinetic parameters of 4E10 and 4G2 scFv proteins in comparison to those of MLS128 mAb. The results demonstrated that 4E10 and 4G2 scFv proteins have a K_D of 10^{-7} M, whereas MLS128 mAb has 10 times the affinity of the scFv proteins due to its slower dissociation rate. As a control, SPR analyses were carried out with MLS128 mAb (Fig. 4C). As seen in Table V, the kinetics parameters obtained were very similar to those described by Osinaga *et al.* (*7*) although their Tn3 peptide differs from the current one in terms of its peptide sequences. This is consistent with the fact that MLS128 mAb recognizes the Tn3 moiety by itself.

Binding activities of scFv proteins to Tn-peptides and natural glycoproteins

Carbohydrate binding abilities of purified 4E10 and 4G2 scFv proteins, originally derived from phage antibodies which were screened against Tn3-peptide, were evaluated together with MLS128 mAb by ELISA. Figure 5A illustrates representative results for the binding of three antibodies to Tn3-, Tn2- and Tn0-peptides. Both 4E10 and 4G2 scFv proteins apparently showed an equal or even higher affinity for Tn2-peptide than Tn3-peptide. In contrast, MLS128 was found to have higher affinity for Tn3-peptide than Tn2-peptide, as was expected (*7*). These results

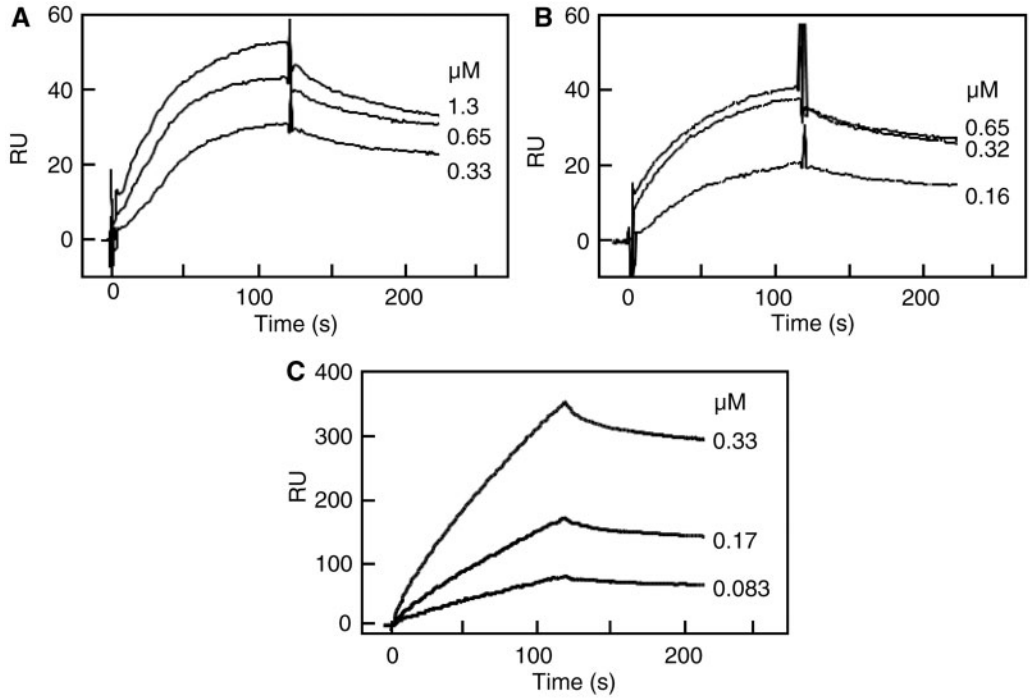

Fig. 4 Binding kinetics of 4E10 and 4G2 scFv proteins and MLS128 mAb by SPR analyses. Sensorgrams of the binding of antibodies, 4E10 and 4G2 scFv proteins (**A** and **B**, respectively), and MLS128 mAb (**C**) to the immobilized Tn3-peptide (NH_2-free) on a CM3 sensor chip. Purified antibodies were injected at the indicated concentrations.

Table V. Kinetic parameters of 4E10, 4G2 and MLS128 binding to Tn3-peptide.

Antibodies	*k*a ($M^{-1}s^{-1}$)	*k*d (s^{-1})	K_D (M)
4E10 scFv	7.7×10^4	2.9×10^{-2}	3.7×10^{-7}
4G2 scFv	1.5×10^4	2.1×10^{-2}	1.4×10^{-7}
MLS128	9.4×10^4	2.0×10^{-3}	2.1×10^{-8}
MLS128[a]	4.5×10^4	1.1×10^{-3}	2.4×10^{-8}

[a]Osinaga *et al.* (*7*): the data were obtained using a different type of Tn3-peptide as ligand, Lys-$(Gly)_4$-Ser(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-$(Gly)_3$.

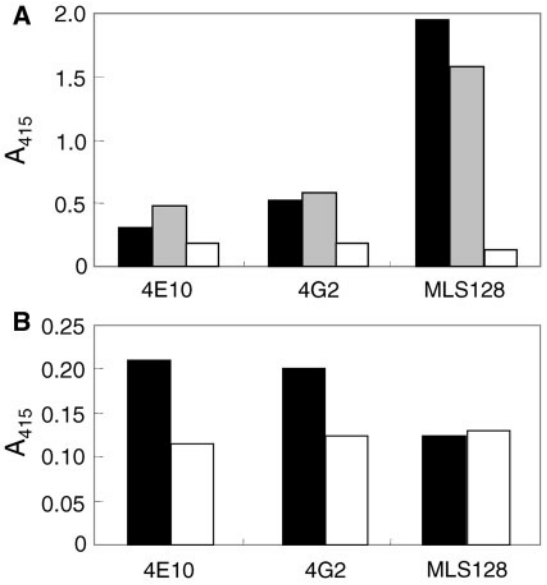

Fig. 5 Binding abilities of purified 4E10 and 4G2 scFv proteins to Tn3- or Tn2-peptide and a natural glycoprotein compared to those of MLS128 mAb. (**A**) Tn3- (closed column), Tn2-(shaded column), and Tn0- (open column) peptides. (**B**) IgA_1 (closed column), BSA (open column).

were also confirmed by SPR analyses (manuscript in preparation).

Since the IgA_1 molecule contains *O*-glycan carbohydrate chains in its hinge region (*9*), binding activities of the three antibodies to IgA_1 were examined. As shown in Fig. 5B, 4E10 and 4G2 scFv proteins bound to IgA_1 whereas MLS128 did not. The results suggested that 4E10 and 4G2 scFv proteins, although derived from phage antibodies isolated using Tn3 as an antigen, seem to bind to Tn2 equally or more preferably, a fact that may be reflected by their different binding abilities to IgA_1 in comparison to MLS128 mAb.

Immunofluorescence staining of cancer cells

4E10 and 4G2 scFv proteins as well as MLS128 mAb were examined for their binding to LS180 and MCF-7 cells, which are known to aberrantly express various types of mucins, including MUC1 and MUC2 (*15–17*). MLS128 mAb was previously shown to bind to those cancer cell lines by fluorescence-activated cell sorting (*8*). Immunofluorescence staining of cancer cells revealed that both 4E10 and 4G2 scFv proteins and MLS128 mAb bound well to LS180 cells (Fig. 6A, C and E, respectively) and to MCF-7 cells (Fig. 6B, D and F, respectively). These results indicated that, similar to MLS128 mAb, both 4E10 and 4G2 scFv proteins bound to extracellular membrane-associated mucins, which are abundant in LS180 and MCF-7 cells.

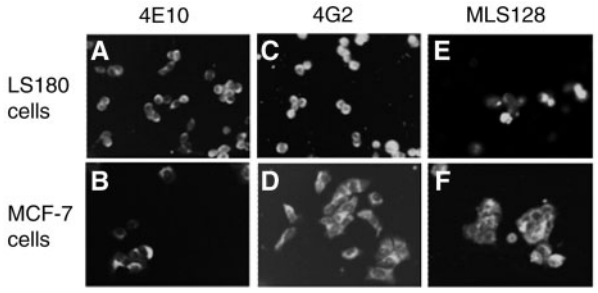

Fig. 6 Immunofluorescence staining of Tn-expressing LS180 and MCF-7 cells with anti-Tn antibodies. Cancer cells were grown on a microscope slide, fixed, and stained with 4E10 scFv (**A** and **B**) or 4G2 scFv (**C** and **D**) or MLS128 mAb (**E** and **F**), followed by anti-MAT tag antibody and Cy3-conjugated anti-mouse IgG Ab as described in 'Materials and Methods' section. Shown is the immunofluorescence staining of LS180 cells (A, C and E) and MCF-7 cells (B, D and F) with magnification of ×60.

Discussion

The aim of this study was to produce human anti-Tn3 antibodies that can be developed into cancer therapeutics based on a recent observation that MLS128 mAb, which has high affinity and specificity for Tn3 (*6*, *7*), inhibits colon and breast cancer cell growth *in vitro* (*8*). In general, anti-carbohydrate antibodies cannot be readily produced *in vivo* since carbohydrates are self-antigens. Anti-carbohydrate antibodies generated by hybridoma technologies often resulted in production of the IgM class and mAbs with low affinities. Phage display technologies can provide anti-carbohydrate antibodies as well as genes encoding them, which can then be readily used to further improve their affinities and also to design novel antibody therapeutics. *In vitro* production of anti-carbohydrate Abs by phage display, however, has resulted in isolation of Abs with low affinities due to the nature of its monovalent display. Several approaches to improve binding for carbohydrate antigens, such as an introduction of a multivalent system and engineering of VH CDR3 to enrich the library, have been reported (*18*–*20*). Previously, anti-trimannose and anti-T-antigen antibodies with high affinity were isolated from an original phage library displaying human scFvs using neoglycolipids as target antigens (*14*, *21*, *22*). Unlike the previously published study, this study used a new phage library with several improvements and synthetic glycopeptides instead of glycolipids.

Two independent scFv genes have been isolated by panning and screening using Tn3-peptide as a target antigen and Tn0-peptide as a control. Sufficient scFv proteins were purified from phagemid-infected *E. coli* to carry out the initial characterization of scFv proteins expressed. Previously, after isolation of phage antibodies, only crude scFv proteins were able to be prepared from phage-infected *E. coli* (*14*). This hampered characterization of isolated phages to identify candidate clones in the initial stage. In contrast, the use of a commercially available pCANTABE5E phagemid allowed purification and thus measurement of affinities for insulin-like growth factor receptor of scFv proteins derived from 1H7 and 3B7 mAbs (*23*). Thus, the new phage library has resolved this major problem associated with the previously used library, indicating the benefit of using the new phage library over the previously used library.

MLS128 mAb (IgG_3) showing greater affinity for Tn3 than for Tn2 was derived from a mouse immunized with LS180 cells (*5*–*7*). Osinaga *et al*. reported that 83D4 mAb (IgM), which was derived from a mouse immunized with cell suspensions obtained from formalin-fixed paraffin-embedded sections of an invasive human breast carcinoma (*24*), shows similar affinity for both Tn2 and Tn3 (*7*). An extremely interesting finding is that both MLS128 and 83D4 mAbs were identified to be anti-Tn antigen antibodies that share similar specificity with a subtle difference, i.e. $Tn3 > Tn2$ and $Tn2 \approx Tn3$, respectively, although they were independently produced. The same group reported production and characterization of mouse/human chimeric antibodies (*25*). IgM*k* was constructed and its parental 83D4 had similar K_D values of 3.1 to 5.1×10^{-9} M for Tn3 and Tn2. Ando *et al*. (*26*) reported anti-tumour effects of mouse–human chimeric anti-Tn IgG_1 against Jurkat cells using KM3413 mAb (IgG_1) derived from a mouse immunized with culture supernatants of LS180 cells. Although the affinity of the chimeric Ab was not shown, the K_D of KM3413 mAb for Tn3-biotin was noted to be 1.6×10^{-7} M, which is similar to those of 4E10 and 4G2 scFv proteins and one-order weaker than that of MLS128 (Tables III and IV). Based on their ELISA results, KM3413 mAb seems to have similar affinities for both Tn2 and Tn3. Unlike previous studies which used cancer cells (*24*, *25*), Danussi *et al*. (*27*) produced mAb 2154F12A4, anti-Tn IgM, by immunizing a mouse with Tn-antigen cluster-conjugated BSA as antigen and screening with the antigen and MCF-7 cells. This mAb was selected on the basis of high binding ability to MCF-7 cells and was then shown to bind MCF-7 tumour lesions as well as metastases in lymph nodes in nude mice. Affinity measurements of this mAb by either ELISA or SPR were not reported, so thus its specificity and affinity are unknown. In the current study, six phage clones displaying human scFvs were isolated by monitoring S/N ratios of their binding to Tn3/Tn0 peptides. Unlike anti-trimannose scFvs, whose amino-acid sequences are quite diverse (*14*), the sequences of six anti-Tn3 scFvs exhibited a high degree of homology, and yet the clones seem to have derived from independent phages. Anti-Tn3 4E10 and 4G2 scFv proteins expressed in *E. coli* were successfully purified to near homogeneity. 4E10 and 4G2 scFv proteins had a K_D of 10^{-7}M for Tn3, which is considered to be a high affinity for carbohydrate-specific monovalent antibodies. In the end, however, both scFv proteins bound to Tn2 with equal or higher affinity as determined by ELISA (Fig. 5A). Tn3 is thus not likely to be an exact epitope for the scFv proteins, suggesting that their specificity differs from that of MLS128. This was also confirmed by the results from ELISA with IgA_1. The current authors previously found that MLS128 only bound asialo-bovine salivary gland mucin (BSM) when western blotting IgA_1, BSM and asialo-BSM with MLS128

(unpublished observations). ELISA results for MLS128 shown in Fig. 5B are consistent with those of western blotting. In contrast, both scFv proteins bound to IgA_1 (Fig. 5B). These results indicate that the scFv proteins do not seem to have the same Tn3 specificity as MLS128, suggesting that the original aim of isolating anti-Tn3 scFvs was not achieved. It is now clear that MLS128 is very unique in recognizing Tn3 since anti-Tn antibodies produced thus far, including the authors' scFv proteins, appear to have equal or greater affinity for Tn2. Although they do not have exactly the same specificity as that of MLS128, the newly isolated scFv proteins that were shown to bind to LS180 and MCF-7 cells are considered to have substantial therapeutic potential. Detailed analyses of the specificity and affinity of 4E10 and 4G2 scFv proteins will be required for development of future antibody therapeutics. Expression and purification of large quantities of 4E10 and 4G2 proteins are underway according to recently established methods for isolation and denaturation of inclusion bodies from *E. coli* over-expressing anti-T antigen-specific scFvs (*22*), followed by affinity purification and refolding (manuscript in preparation). Furthermore, CHO cell clones stably expressing and secreting 4E10 scFv–Fc proteins have been isolated, which should allow purification of a substantial amount of scFv–Fc proteins and further analyses of their affinity and specificity as well as growth-inhibitory effects.

Previous publications on anti-Tn antibodies described the development and some uses of mouse/human chimeric antibody (*25*), mouse CDR-grafted human antibody (*26*), or mouse mAb (*27*) in cancer diagnostics and/or therapeutics. Since anti-Tn cluster scFv antibodies described in this article are the first fully human antibodies, these antibodies can be readily developed into cancer therapeutics. In summary, this study not only described the successful isolation of two human-type scFv proteins with a high level of affinity for Tn2 and Tn3 from a new phage library, but it also indicated the great potential for development of antibody therapeutics targeting colon and breast cancer.

Acknowledgements

The authors wish to thank Drs Iwase and Nakada for providing human IgA_1 and MLS128 mAb, respectively.

Funding

This work was supported by a grant from Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST). Dr Ayano Takasaki-Matsumoto is a recipient of the RPD from the Japan Society for the Promotion of Science (JSPS).

Conflict of interest

None declared.

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