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Construction and expression of anti-Tn-antigen-specific single-chain antibody genes from hybridoma producing MLS128 monoclonal antibody

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Noriyuki Yuasa[1], Haruhiko Ogawa[1], Tomoyuki Koizumi[1], Kazuhiro Tsukamoto[1], Ayano Matsumoto-Takasaki[1,2], Hideki Asanuma[3], Hiroshi Nakada[4] and Yoko Fujita-Yamaguchi[1,*]

[1]Department of Applied Biochemistry, Tokai University School of Engineering, Kanagawa, Japan; [2]Division of Tumor Cell Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA; [3]Center for Influenza Virus Research, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama-shi, Tokyo 208-0011; and [4]Department of Molecular Bioscience, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan

*Yoko Fujita-Yamaguchi, Department of Applied Biochemistry, Tokai University School of Engineering, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan. Tel: +81 463 58 1211 (ext. 4188), Fax: +81 463 50 2012, email: yokoyamaguchi@tokai-u.jp

Anti-Tn-antigen monoclonal antibody MLS128 has affinity for three consecutive Tn-antigens (Tn3) more than Tn2. The major aim of this study was to isolate genes encoding MLS128 variable domains to produce a large quantity of recombinant MLS128 antibodies, in turn, allowing the conduct of studies on precise interactions between Tn3- or Tn2-epitopes and MLS128. This study describes cloning of the variable region genes of MLS128, construction of the variable region genes in single-chain variable fragments (scFv) and two scFvs conjugated with human IgG_1 hinge and Fc regions (scFv–Fc) types, and their respective expression in bacterial and mammalian cell. MLS128 scFv protein with the expected specificity and affinity was successfully prepared from inclusion bodies accumulating in *Escherichia coli*. Construction, expression and purification of two types of MLS128-scFv–Fc proteins with differing linker lengths in Chinese hamster ovary cells demonstrated that the purified scFv–Fc proteins had binding activity specific to the glycoprotein-expressing Tn-antigen clusters. These results revealed that VL and VH genes cloned from the hybridoma represent those of MLS128 and that recombinant antibodies produced from these genes should provide sufficient amounts of binding domains for use in 3D structural studies such as NMR and X-ray analysis.

Keywords: antibody engineering/phage display/protein refolding/single-chain Fv/Tn-antigen.

Abbreviations: Ab, antibody; ABTS, 2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; CDR, complementarity determining region; CBB, Coomassie Brilliant Blue; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Gdn–HCl, guanidine hydrochloride; GPA, Glycophorin A HRP, horseradish peroxidase; IPTG, isopropyl β-D thiogalactoside; mAb, monoclonal Ab; IMDM, Iscove's Modified Dulbecco's Medium; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; scFv, single-chain variable fragment; TBS, Tris buffered saline; TMB, 3,3′,5,5′-Tetramethylbenzidine; Tween 20, Polyoxyethylene Sorbitan Monolaurate; VH, variable region of antibody heavy chain; VL, variable region of antibody light chain.

Glycopeptide antigens represent attractive targets for diagnosis, vaccination and immunotherapy for cancers (*1*). Tn-antigen, GalNAcα-Ser/Thr, is one such antigen associated with carcinomas. Tn-antigen provides a sensitive and specific marker for pre-clinical detection of carcinoma and represents a potential therapeutic target for cancer treatment (*2–5*). There are several reports of monoclonal antibodies with specificity for Tn-antigen that serve as histological and diagnostic reagents (*6–12*). Anti-Tn-antigen monoclonal antibody MLS128, derived from a mouse immunized with LS180 human colon carcinoma cells (*13*), is IgG_3 that recognizes the structure of three consecutive Tn-antigens (Tn3) (*14*, *15*). The current authors reported that MLS128 treatment significantly inhibited colon and breast cancer cell growth and that MLS128 treatment caused down-regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor in LS180 cells (*16*).

The structural information on the recognition sites of Tn-antigen-specific antibodies is extremely limited; however, that on lectins is available (*16–18*). MLS128 is unique in the sense that it is known to be Tn3-specific (*14*, *15*). Other Tn-antigen specific mAbs thus far reported exhibit Tn2-binding activity (*15*, *19*). However, by thoroughly examining the kinetics of MLS128 binding to three and two consecutive Tn-antigens using Tn3- and Tn2-peptides, respectively, at different temperatures, we recently found that Tn3 specificity is temperature-dependent (*31*). Cloning of cDNAs encoding MLS128 VL and VH represents the next important step in deciphering detailed

interactions between the binding domain of MLS128 and the Tn3 or Tn2 epitope.

In the current study, the variable region genes of MLS128 were cloned. MLS128 antibody genes were constructed in scFv and two scFv–Fc forms with different linker lengths, and their expression was ascertained in bacterial and mammalian cells, respectively. As a result, MLS128 scFv protein with the expected specificity and affinity was successfully prepared with high yields from inclusion bodies accumulating in *Escherichia coli* by denaturation, purification and refolding processes. Moreover, the construction, expression and purification of MLS128-scFv–Fc proteins in two forms with differing linker lengths were accomplished in Chinese hamster ovary (CHO) cells. MLS128 scFv–Fc proteins purified from the supernatant of stably transfected CHO cells showed specific binding activity to glycoprotein-expressing Tn-antigen clusters. These studies thus revealed that VL and VH genes cloned from the hybridoma represent those of MLS128 monoclonal antibody. The recombinant MLS128 antibodies produced from these genes should provide sufficient amounts of binding domains as well as stable-isotope labelling that are definitely required for NMR analyses, of which significant progress has already been made (*20*). This study has laid the foundation for intensive analyses of Tn3- or Tn2-ligand and MLS128.

Materials and Methods

Materials

The murine hybridoma cells that secret monoclonal antibody MLS128 against Tn-antigen were produced as previously described (*13*). *Escherichia coli* JM109 component cell, DNA polymerase and restriction enzyme were purchased from TaKaRa (Shiga, Japan). *Escherichia coli* TG1 and the non-suppressor strain Top10F′ were purchased from Invitrogen (Carlsbad, CA). Helper phages M13KO7, anti-M13 antibodies, anti-E-tag antibodies, Ni^{2+}-Sepharose and pCANTAB 5E were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St Louis, MO). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Roche Diagnostics (Mannheim, Germany). A DAB detection kit was purchased from Vector Laboratories (Burlingame, CA). Carbenicillin and Kanamycin were purchased from Wako Pure Chemical (Osaka, Japan). Benzonase was purchased from Novagen (Darmstadt, Germany). Covalink 96-well plates were purchased from Nunc (Roskilde, Denmark). B-PER Bacterial Protein Extraction Reagent was purchased from Pirece Protein Research (Rockford, IL). Horseradish peroxidase (HRP)-conjugated anti-6×His tag antibody was purchased from Abcam (Cambridge, UK) Synthetic Tn3-, Tn2- and Th0-peptides summarized in Table I were prepared as described (*21*).

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

Tn peptides	Structure
Tn0	Gly-Thr-Thr-Thr-βAla-Gly-OH
Tn2	Gly-Thr-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH
Tn3	Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH

Cloning of cDNAs Encoding VL and VH Domains of MLS128

Construction of MLS128 scFv Phage. Total RNA was isolated from MLS128-secreting hybridoma cells with a TRIzol reagent (Invitrogen Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using the ImProm-II reverse transcription system (Promega, Madison, WI). MLS128 variable heavy (VH) and variable light (VL) chain gene fragments were amplified by PCR using various combinations of primer sets according to previously described methods (*22*). The amplified VH and VL purified by agarose gel electrophoresis were subjected to assembly PCR. Briefly, equal amounts of VH and VL fragments were mixed and PCR carried out in 10 cycles with denaturing at 94°C for 10 s, annealing at 55°C for 30 s and extension at 68°C for 1 min, followed by second round of PCR for 30 cycles using a primer set (sense : 5′-GAGGAGGAGGCGGGGCCCAGCCGG CCGA-3′, antisense : 5′-GAGGAGGAGGCGGCCGCATAGTGA CA-3′) with denaturing at 94°C for 10 s, annealing at 55°C for 30 s and extension at 68°C for 1 min. After gel purification, the PCR products were digested with *Sfi*I and *Not*I and ligated into a phagemid expression vector, pCANTAB 5E (GE healthcare Piscataway, NJ). The ligation mixture was purified and electroporated into *E. coli* TG1 cells.

Expression of Phage Antibodies. Infected *E.coli* TG1 cells were grown in 40 ml of LB containing 50 μg/ml carbenicillin and 2% glucose at 30°C for 16 h with shaking. Phages were rescued after addition of 40 μl containing 3.5×10^9 pfu of M13KO07 helper phages and incubation at 37°C for 1 h without shaking, followed by addition of kanamycin (50 μg/ml) and incubation at 25°C for 40 h with shaking. The culture supernatants containing phages were precipitated by adjusting to final concentrations of 4% polyethylene glycol and 0.5 M NaCl. Pellets were suspended in TBS (20 mM Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl) containing 1.5% BSA and 0.2% Tween 20, followed by treatment with benzonase (Novagen) to digest any unnecessary DNA. The titre was determined for each phage by means of infection activity against *E. coli* TG1 cells.

Production of Soluble scFv Proteins, Western Blotting and DNA Sequencing. To produce soluble scFv antibodies for initial characterization, the phagemid DNA was used to transform *E. coli* Top10F′. This strain of *E. coli* recognizes the amber stop codon between the scFv and the gene III fragment. *Escherichia coli* cells were cultured overnight in 5 ml of LB medium containing 50 μg/ml carbenicillin and 1% glucose at 25°C. One millilitre of the overnight culture was inoculated in 40 ml of the fresh LB medium and incubated in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 20°C for 16 h. The periplasm fraction recovered in an osmotic shock buffer was clarified by centrifugation at 12,000*g* for 15 min and dialysed against PBS. Levels of scFv protein expression were examined by Western blotting of the periplasmic extracts using mouse anti-E tag antibody (GE Healthcare) and HRP-conjugated anti-mouse IgG(H+L) antibody (Jackson ImmunoResearch) as primary and secondary antibodies, respectively. DAB staining kit (Vector Laboratories, Inc., CA) was used for colour development.

DNA sequencing of scFvs constructed from selected clones was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems) using S1 and S6 sequencing primers for the pCANTAB 5E vectors (GE Healthcare). Deduced amino acid sequences of VH and VL chains derived from the hybridoma were compared with known immunoglobulin amino acid sequences in the Kabat database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Purification of MLS128 scFv Expressed in E. coli Cells

Construction of the MLS128-scFv Bacterial Expression Vector. Oligonucleotide primers MLS-f and MLS-r were designed to add *Eco*RI site to the 5′ end of MLS128 scFv and to add *Not*I site to the 3′ end, MLS-f: 5′-CATGAATTCCGATATTGTGTTGACACA GTCTCACAAATTC-3′, MLS-r: 5′-TGCGCGGCCGCACCTTGG CCCCAGTAGTCC-3′. Of six independent clones isolated, MLS128-F11 phagemid was used as a template for construction of MLS128 scFv. PCR was performed with denaturation at 94°C for 2 min, 30 cycles of amplification at 94°C for 30 s, at 59°C for 1 min, and 72°C for 1 min. PCR products were digested by *Eco*RI/*Not*I restriction enzymes and cloned into the pET22b(+) vector, which was transformed into *E. coli* JM109 for propagation. DNA sequence analysis of the scFv insert was performed using T7 promoter and T7

terminator primers. The resulting expression vector, pET22-MLS128 scFv, was transformed into competent *E. coli* BL21(DE3) cells for expression of MLS128 scFv protein.

Preparation of Inclusion Bodies from E.coli *Cells Overexpressing MLS128 scFv Proteins*. *E. coli* BL21(DE3) cell transformed with pET22b-MLS128 expression vector were inoculated into 40 ml of LB medium containing 50 μg/ml carbenicillin. After cells were grown to OD600 of ~0.6 by incubation at 37°C with shaking, expression of scFv proteins was induced by the addition of 1 mM IPTG into the medium. After incubation for 4 h, bacterial cells were harvested by centrifugation at 2,900*g* for 30 min and stored at −30°C for future experiments.

Inclusion bodies were prepared from the bacterial cells by suspending in B-PER Bacterial Protein Extraction Reagent (Pierce; Thermo Scientific Life Science, Rockford, IL) according to the manufacture's protocol. Briefly, 2.5 ml of the reagent containing 500 μg of lysozyme and 63 U of benzonase per 1 g of wet cells were added to bacterial cells. After gentle shaking for 10 min, pellets were collected by centrifugation at 14,500*g* for 20 min and suspended in 15 ml of 10-fold diluted B-PER reagent. After gentle shaking for 10 min, pellets were collected as inclusion bodies by centrifugation at 14,500*g* for 20 min.

Purification and Refolding of MLS128 scFv Proteins from Inclusion Bodies. Inclusion bodies were solubilized in 20 mM sodium phosphate buffer, pH 7.4, containing 3.5 M Gdn–HCl and 0.2 M NaCl (3.5 M Gdn–HCl buffer) and subjected to centrifugation at 14,500*g* for 30 min. Ni^{2+}-Sepharose gels (0.5 ml) were added to a test tube containing the supernatant. After the tube was inverted overnight at 25°C, the Ni^{2+}-Sepharose gels were packed into a column. The column was washed thoroughly with 3.5 M Gdn–HCl buffer containing 40 mM imidazole. ScFv proteins were then eluted with 3.5 M Gdn–HCl buffer containing 500 mM imidazole and 5 mM DTT.

Refolding of purified scFv proteins in the presence of 3.5 M Gdn–HCl was carried out with some modifications according to the stepwise dialysis method previously described (*23*). The concentrations of the unfolded scFv proteins were adjusted to 3.3 μM in the 3.5 M Gdn–HCl buffer. The affinity-purified denatured scFv proteins in 20 ml were dialysed against 200 ml of 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl and 1 mM EDTA (dialysis buffer) in the presence of 2 M Gdn–HCl then (i) 40 ml of the dialysis buffer containing 1 M Gdn–HCl, 0.4 M L-arginine, 1 mM GSSG, and 2 mM GSH; (ii) 40 ml of the dialysis buffer containing 0.5 M Gdn–HCl, 0.4 M L-arginine, 1 mM GSSG, and 2 mM GSH; (iii) 200 ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl).

Production and Purification of Two MLS128 scFv–Fc Constructs Expressed in CHO Cells

Construction of Two Forms of MLS128-scFv–Fc Expression Vectors. The MLS128 scFv gene constructed as described above was inserted into a template plasmid signal-human Fc-pCI-neo (pCI-Fc) to stably express scFv–Fc proteins in dihydrofolatereductase-deficient Chinese hamster ovary (CHO-DHFR) cells as previously described (*24*, *25*). Briefly, oligonucleotide primers, Fc-fw and Fc-rev, were designed to respectively add the *Eco*RV site to the 5′ end of MLS128 scFv and the *Xba*I site to the 3′ end: VL-fw: 5′-GACGATATCGTGTTGACACAGTCTCAC-3′, VH-rev: 3′-GCA TCTAGAGGCTGAGGAAGACTGTGAGAGT-3′. PCR was performed using the MLS128-F11 phagemid as a template with denaturation at 94°C for 2 min and 30 cycles of amplification at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. PCR products were digested with the restriction enzyme *Eco*RV and cloned into the *Eco*RV site of pCI-Fc vector and transformed into *E. coli* JM109 for propagation. Afterwards, the same PCR products were digested with the restriction enzyme *Xba*I and cloned into the *Xba*I site of the intermediate vector and transformed into *E. coli* JM109 for propagation.

As summarized in Fig. 1, phage-derived MLS128 scFv and scFv–Fc contained a short linker, GGSSRSS. Thus, this was named MLS128 scFv–Fc (S). Since the length of linker may cause problems in self-folding of the binding domain, MLS128 scFv–Fc with a longer linker containing 12 amino acids named MLS128 scFv–Fc (L) was constructed. Specific oligonucleotide primers were used to amplify VH gene segments from the purifiedMLS128-F11 phagemid: VH fw-L, 5′-AGCTCTAGATCT GGAGGTGGCGGTTCAGGTGGAGGAGGTTCCCTCGAGGT TCAGCTGCAGCAG-3′, and VH-rev. The purified PCR product encodes a 12-amino acid peptide linker (RSGGGGSGGGGS). The DNA fragments were digested with the restriction enzyme *Xba*I and cloned into the *Xba*I site of the intermediate vector. The resulting pCI-MLS scFv–Fc (L) vector was transformed into *E. coli* JM109 for propagation. The sequences of resulting vectors were confirmed by DNA sequencing. The expression vectors were purified by QIAGEN EndoFree Plasmid Maxi and stored in sterile water to be used for lipofection into mammalian cells.

Cloning of CHO Cells Stably Expressing MLS128 scFv–Fc Proteins. CHO-DHFR cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FBS, 100 μM hypoxanthine, 16 μM thymidine and Penicillin-Streptomycin (Sigma-Aldrich). pCI-MLS128 scFv–Fc (S) and pCI-MLS128 scFv–Fc (L) were transfected into CHO-DHFR cells using Lipofectamine 2000 reagent (Invitrogen) in serum-free OPTI-MEM (GIBCO BRL, Grand Island, NY) medium in general accordance with the manufacturer's instruction. Cell lines stably expressing scFv–Fc proteins were established by growing CHO cells in the presence of G418 (400 μg/ml) and subjected to these cells to cloning by limited dilution methods. Levels of scFv–Fc protein expression were quantified by sandwich ELISA. Briefly, goat anti-human IgG (Fc fragment specific, Jackson Immuno Research) diluted in phosphate-buffered saline (PBS) to a final concentration of 5 μg/ml was coated in wells of 96-well plates by adding 100 μl/well and incubating the plates overnight at 4°C. Plates were blocked with 3% BSA in PBS and then, cell culture supernatants from different clones were added and incubated at 37°C for 1 h. After plates were washed with PBS, HRP-conjugated anti-human IgG (Jackson Immuno Research) diluted to 1:1,000 was added to the wells. The wells were washed with PBS and then incubated with ABTS (Roche Diagnostics GmbH, Mannheim, Germany). The colour development in the wells of plates was measured at 405 nm with a Bio-Rad Microplate Reader.

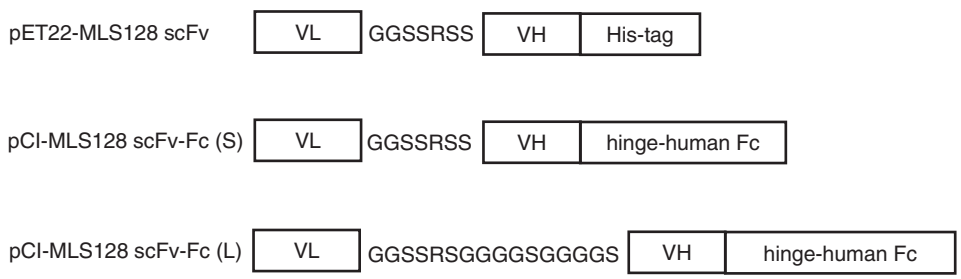

Fig. 1 Schematic representation of MLS128 scFv and scFv–Fc constructs described in this study. The VL, VH and additional domains are shown as boxes: VL, variable domain of the light chain of the mouse MLS128 monoclonal antibody; VH, variable domain of the heavy chain of the mouse MLS128 monoclonal antibody; His-tag, 6-histidine epitope sequence; hinge-human Fc, Hinge and CH2-CH3 region of the human IgG_1antibody.

Purification of MLS128 scFv–Fc Proteins from Culture Media. The establish clones were cultured in T175 flasks (Corning, Inc., Corning, NY) for 72–84 h. Culture supernatants were passed through a Protein A-Sepharose column equilibrated with 50 mM Tris–HCl, pH 7.5, the column was washed with 5 column volumes of the same buffer. After contaminating, bovine IgG was eluted from the column with 15 column volumes of 50 mM citric acid buffer, pH 5.0, scFv–Fc proteins were eluted from the column with 10 column volumes of 50 mM citric acid buffer, pH 3.0, containing 50 mM NaCl. The eluted scFv–Fc proteins were immediately neutralized by adding 0.17 volume of 1 M Tris–HCl, pH 9.0. The eluates were concentrated and dialysed against PBS using Amicon Ultra-15 (Millipore). Protein concentration was determined using Bradford method with BSA as a standard.

Characterization of Purified MLS128 scFv and MLS128 scFv–Fc Proteins

SDS–PAGE and Western blotting. The purified scFv and scFv–Fc proteins were analysed with SDS-PAGE (5–20% gradient polyacrylamide gel) under reducing or non-reducing conditions. Separated proteins were stained with Coomassie Brilliant Blue (CBB). For immunoblotting, the proteins on the SDS–PAGE gel were transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with PBS containing 3% BSA for 2 h at room temperature. After washing with PBS, the membrane was incubated with HRP conjugated anti-His-Tag antibody or HRP-conjugated anti-human Fc antibody at room temperature for 1 h. The membrane was washed with PBS containing 0.2% Tween and PBS five times each. Proteins were visualized by colour development using the DAB staining kit (Vector Laboratories, Inc., CA).

Tn-antigen-binding Activity of MLS128 scFv According to ELISA. The binding activity of MLS128 scFvs or those expressed by phages to Tn3-peptide was measured using ELISA. Tn3 and Tn0-peptides were covalently linked to the free amino groups on Covalink 96-well plates via carboxyl groups using EDC as a coupling reagent. Briefly, 25 µl of 50 µM Tn3 peptide and 2.5 mM EDC in water were added to Covalink plate wells and allowed to react for 2 h at 37°C. After plates were blocked with 3% BSA/TBS for 1 h at 37°C, wells were incubated with phage antibodies or scFvs for 1 h at 37°C. The wells were washed three times with TBS containing 0.05% Tween 20 (TBST). Bound phages were detected by incubation with a 1:1,000 dilution of rabbit anti-M13 antibody, followed by incubation with HRP-conjugated anti-rabbit IgG (1:1,000 dilution) for 1 h at 37°C. Detection was achieved by colour development after the addition of ABTS substrate (Roche Diagnostics). For detection of soluble MLS128 scFvs, a mouse monoclonal antibody to the E-tag was used as a second antibody. After washing, MLS128 scFvs were detected by incubation with HRP-conjugated anti-mouse IgG(H+L) antibody (Jackson). The DAB staining kit (Vector Laboratories, Inc., CA) was used for colour development.

ELISA similar to that described earlier was performed to determine the antigen-binding activity of purified MLS128 scFv and scFv–Fc proteins as well as authentic MLS128. Tn3, Tn2 and Tn0 peptides were covalently linked to the free amino groups on Covalink 96-well plates via carboxyl groups using EDC as a coupling reagent. 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-conjugated His-probe and HRP-conjugated anti-mouse IgG antibody for scFv proteins and MLS128 mAb, respectively, for 1 h at room temperature. The wells were washed five times with TBST and then five times with TBS. The bound scFv proteins or mAb were detected by colour development using a 100 µl ABTS solution (Roche Diagnostics, Basel, Switzerland). The reaction was stopped by adding 100 µl of 2% oxalic acid. Absorbance at 405 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA).

Determination of Affinity Constants of MLS128 scFv by Surface Plasmon Resonance. Real-time measurements of the binding of MLS128 scFv protein to the Tn-peptides were performed by surface plasmon resonance (SPR) as described elsewhere (*21*). Briefly, all SPR analyses were carried out at 10°C on a BIAcore 3000 biosensor (GE Healthcare, Uppsala, Sweden). N-terminus free Tn3-, Tn2- 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 the Tn-antigens 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 MLS128 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.

Reactivity of MLS128 scFv–Fc Proteins to Glycophorin A and Asialo- and Asialo-agalacto-Glycophorin A. Glycophorin A (GPA) contains major O-linked oligosaccharide structures that have been extensively characterized (*26*). On the basis of a previous report in which GPA peptides isolated by HPLC were treated with neuraminidase and β-galactosidase after being immobilized on plastic plates for determination of specificity of MLS128 (*14*), asialo- and asialo-agalacto-GPA were recently prepared in solution for ready use in the detection of MLS128-binding activity (*27*). GPA and asialo- and asialo-agalacto-GPA were plated in wells of 96-well plates (1.35 µg/well). After incubation at room temperature for 1 h and removal of unbound GPA proteins, the wells were blocked by incubation with 50 mM Tris–buffer saline, pH 7.4 (TBS), containing 9% perfect block at 37°C for 1 h. Purified MLS128 scFv–Fc proteins at different concentrations were added to wells and allowed to react to Tn-antigens for 1 h at room temperature. After wells were washed with 200 µl TBS five times, the bound MLS128 scFv–Fc proteins were incubated with 50 µl of 1,000-fold diluted HRP-conjugated anti human Fc antibody at room temperature for 1 h. The wells were washed with 200 µl TBS five times. The bound HRP was detected using TMB (3,3′,5,5′-tetramethylbenzidine, Kierkegaard & Perry Laboratories Inc., MD) as a substrate. After incubation in the dark for 30 min, absorbance at 655 nm was measured using a plate reader. As a control, binding of MLS128 to GPA and asialo- and asialo-agalacto-GPA was determined using ELISA. After binding and washing were done as described above, 50 µl of 1,000-fold diluted anti-mouse IgG-HRP were added and the plate was incubated at room temperature for 1 h. The bound HRP activity was measured as described above.

Results

Cloning of cDNAs Encoding VL and VH Domains of MLS128

Isolation of VH and VL cDNA and Characterization of Phage-displayed MLS128 scFvs. VH and VL cDNA from a hybridoma cell line producing MLS128 were prepared by PCR using various combinations of primers as previously described (*22*), and VH and VL were assembled into scFvs in a VL-linker-VH form. The scFv DNA fragments were isolated, purified, digested and then ligated into pCANTAB 5E in front of the E-tag DNA fragment. The resulting phagemids were introduced into *E. coli* TG1 cells (amber suppression strain), in which the amber stop codon between the E-tag DNA sequence and fd gIII was read through, allowing production of scFv E-tag-pIII fusion proteins. Randomly isolated *E. coli* TG1 clones were analysed using PCR amplification and restriction digestion with *Sfi*I and *Not*I to determine the presence of the scFv DNA insert in these clones. Phagemid particles displaying scFvs were rescued from *E. coli* cells infected with helper phage M13K07. Phage clones were analysed with ELISA and SPR, which indicated that all of the recombinant phages tested had binding affinity for Tn-3 peptide (data not shown).

Production and Characterization of Soluble scFv Proteins. Expression of scFv-E-tag fusion proteins was induced by incubation of *E. coli* Top10F′cells with 1 mM IPTG at 20°C for 25 h. The preiplasm

fractions of all nine clones examined expressed the scFv protein (~28 kDa) as detected by immunoblotting with anti-E-tag antibody and exhibited Tn3-peptide-binding activity according to ELISA and SPR (data not shown).

Sequence Analysis. Eleven independent clones were chosen for DNA sequencing of their scFv inserts. Clones F11 and three other clones shared the same sequence. The rest of the clones contained nearly the same sequences as those of clone F11 except for a difference of one or two amino acids in FWRs (data not shown). The deduced amino acid sequences of the VH and VL of clone F11 are shown in Fig. 2, where the VH and VL amino acid sequence of 83D4 (*9*) are included for comparison of the amino acid sequences. On the basis of amino acid sequences, the VH of MLS128 is classified as a mouse heavy-chain subgroup J558(IIB), whereas VL of MLS128 falls into a mouse kappa light-chain subgroup III (*28*). Screening the nucleotide sequences of VH and VL of MLS128 against the respective entries for the Ig heavy chain and Ig kappa light chain in the Kabat Database revealed that the VH sequence was closely related to that of an anti-Tn mAb 83D4, with 97% nucleotide sequence identity. Interestingly, VH domains of MLS128 and 83D4 differed in terms of only 11 nucleotides resulting in three amino acid changes. Among the eight changed nucleotides, a single change in framework region 1 (FWR1) was attributed to the use of a degenerate PCR primer. Three single silent nucleotide changes (resulting in no amino acid sequence changes) occurred in both complementarity-determining regions 2 (CDR2) and FWR3. Four nucleotide changes occurred in CDR3 and caused the five amino acid changes in CDR3 (Fig. 2), which are practically the only discrepancies between VH domains in the two mAb.

Despite the highest level of homology found in the VH of 83D4, VL domains of MLS128 and 83D4 showed only 67% nucleotide sequence identity. Instead, the VL sequence of MLS128 was found to be closely related to that of an anti-blood group A hybridoma A003-40/5G7k (*29*, *30*). There was 98% nucleotide sequence identity between VL of MLS128 and A003-40/5G7k, with only six nucleotide differences that resulted in three amino acid changes. Among the six changed nucleotides, three were found in FWR1 while three others found in FWR4 appeared to be due to the use of a degenerate PCR primer.

Purification of MLS128 scFv Expressed in E. coli Cells

Purification and Refolding of MLS128 scFv Protein from Inclusion Bodies. Initial characterization of phage-displayed MLS128 scFv (F11 clone) as well as the scFv protein confirmed that the gene product of the constructed MLS128 scFv has the Tn3-binding activity, as expected. In order to further characterize the affinity and specificity of the MLS128 scFv protein, however, large-scale purification of an active MLS128 scFv protein was required. Since scFv proteins expressed by the pET-22 b(+) expression vectors contain the pelB signal sequence of 3.4 kDa at the N-termini, they are presumably transported to the periplasm where proper folding is supposed to take place. Initial efforts to express MLS128 scFv in a soluble form, however, failed (data not shown). Thus, as an alternative strategy, involving MLS128 scFv proteins overexpressed and recovered as insoluble proteins in inclusion bodies was used for large-scale purification. Inclusion bodies isolated from IPTG-induced *E. coli* BL21(DE3) cells transformed with the pET22b-MLS128 expression vector were found to contain mostly insoluble MLS128scFv proteins according to SDS–PAGE and CBB staining followed by Western

VH

	FWR1	CDR1	FWR2	CDR2
MLS128	EVQLQQSDAELVKPGASVKISCKASGYTFT	DHAIH	WVKQKPEQGLEWIG	YFSPGNGDIKYNEKFKG
83D4	QVQLQQSDAELVKPGASVKISCKASGYTFT	DHAIH	WVKQKPEQGLEWIG	YFSPGNGDIKYNEKFKG
Consensus	:******************************	*****	**********	**********

	FWR3	CDR3	FWR4
MLS128	KATLTADKSSSTAYMQLNSLTSEDSAVYFCKRS	QPGKWDY	WGQGTTLTVSS
83D4	KATLTADKSSSTAYMQLNSLTSEDSAVYFCKRS	-YGNYDY	WGQGTTLTVSS
Consensus	**********************************	*::**	**********

VL

	FWR1	CDR1	FWR2	CDR2
MLS128	QSHKFMSTSVGDRVSITC	KASQDVSTTVA	WYQQKPGQSPKLLIY	GVPDRFTGS
83D4	QSPAILSVSPGERVSFSC	RASQNIGTSIH	WYQQRTNGSPRLLIK	GIPSRFSGS
Consensus	** ::*.* *:***::*	:***::.*::	****:.. **:***	*:*.**:**

	FWR3	CDR3	FWR4
MLS128	GSGTDFTFTISSVQAEDLAVYYC	QQHYST-----P	PTFGGGTKLELK
83D4	GSGTDFTLSINSVESEDIADYYC	ADYYCQHTNSWP	TTFGGGTKLEIK
Consensus	********::*.**::**:* ***	::*. *	.**********:*

Fig. 2 Amino acid sequences of MLS128 VH and VL domains aligned with those of 83D4. The deduced amino acid sequences of VH and VL domains of MLS128 are shown together with those of 83D4 (*9*) to allow comparison of their amino acid sequences.

blotting with HRP-conjugated anti-His-tag antibody (data not shown). Isolated inclusion bodies were solubilized in 3.5 M Gdn–HCl, from which scFv proteins were purified by Ni^{2+}-Sepharose chromatography in the presence of 3.5 M Gdn–HCl. The purified scFv proteins were refolded by a modified stepwise dialysis method (*23*). Table II shows a typical purification result. Starting with a 40 ml culture, 1.6 mg of soluble MLS128 scFv proteins were recovered as soluble form from the insoluble fractions with a yield of 94%. The resulting soluble scFv proteins moved as a single band with a theoretical molecular mass of 27 kDa and were apparently pure according to SDS–PAGE and CBB staining under reducing conditions (Fig. 3).

Assessments of the Binding Activity of the Purified and Refolded MLS128 scFv Protein. The MLS128 scFv proteins that had been purified and refolded were subjected to ELISA and SPR analyses. Affinity constants of MLS128 binding to Tn3- and Tn2-peptides are temperature-dependent (*31*). ELISA was thus carried out at both 4 and 37°C with the purified MLS128 scFv protein as well as with MLS128 as a control. The results shown in Fig. 4 clearly indicate that MLS128 scFv protein exhibited specific Tn3-binding activity at 4°C (Fig. 4A). When assayed at 37°C, MLS128 scFv protein had greater Tn2-binding activity than Tn3-binding activity (Fig. 4B). This result is consistent with that of MLS128 mAb, as shown in Fig. 4C and D confirming that the recombinant MLS128 scFv protein retains the unique characteristics of MLS128 mAb with respect to Tn3/Tn2 specificity and/or affinity. The results also demonstrated that the affinity of the MLS128 scFv protein for Tn3- or Tn3-peptide was more than an order less than that of its corresponding mAb, as expected (*15*). A point of note, however, is that non-specific binding to Tn0-peptide was significantly higher with the scFv proteins than that with mAb (Fig. 4A, B and C and D, respectively). This indicates the possibility that refolded scFv proteins may have aggregated which tends to cause such a high non-specific background.

Affinity constants of purified and refolded MLS128 scFv proteins with respect to Tn3- and Tn2-peptides were determined by SPR at 10°C. The sensorgrams shown in Fig. 5 clearly demonstrate that Tn3 binds to the scFv protein with higher affinity than Tn2. This result agrees with the binding characteristics of the parental MLS128 mAb. Kinetic parameters for MLS128 scFv protein were determined from the sensorgrams shown in Fig. 5 and are compared with those of the parental MLS128 mAb obtained by Osinaga *et al*. (*15*, panel b) and by Matsumoto-Takasaki *et al*. (*31*, panel c) in Table III.

Table II. Summary of purification and refolding of the MLS128 scFv protein from inclusion bodies prepared from 80 ml *E. coli* culture.

Purification and refolding processes	Protein (μg/ml)	Volume (ml)	Yield (mg)	Ratio of refolded scFv (%)
Solubilization of inclusion bodies with 3.5 M Gdn–HCl	488.1	20	9.76	
Ni^{2+}-Sepharose chromatography eluates in the presence of 3.5 M Gdn–HCl	854.2	2	1.71	100%
Soluble fractions recovered after removing Gdn–HCl and refolding	320.9	5	1.60	94%

Protein concentrations were estimated by Bradford method using human IgG as standard protein.

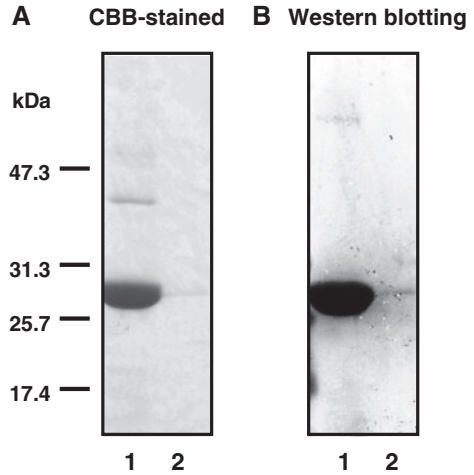

Fig. 3 SDS–PAGE and Western blotting of purified and refolded scFv proteins. ScFv proteins recovered as inclusion bodies were solubilized and purified with Ni^{2+}-Sepharose chromatography in the presence of 3.5 M Gdn–HCl and then refolded by a modified stepwise dialysis method (*23*). After dialysis against PBS, comparable volumes of soluble and insoluble fractions (lanes 1 and 2, respectively) were analysed with SDS–PAGE on 12.5% gel under reducing conditions. (A) Coomassie Brilliant Blue stained gel. (B) Western blotting with anti-His-Tag antibody.

Purification of Two MLS128 scFv–Fc Constructs Expressed in CHO Cells

Production of scFv–Fc Proteins in CHO Cells. Characterization of the VL and VH cDNAs prepared form hybridoma cells producing MLS128 mAb as described above strongly indicated that the isolated genes represent authentic mRNAs. In order to further confirm the authenticity of the cloned VL and VH cDNAs, MLS128 scFv–Fc proteins were also produced in CHO cells as described previously (*24*, *25*). In the mammalian cell system, expressed scFv–Fc proteins will be secreted as soluble form with intact binding activity.

MLS128 scFv-Fc genes with two different linker lengths, as shown in Fig. 1, were constructed to test whether the original short linker consisting of seven amino acids contributed to dimerization of scFvs or caused lower binding activity as observed with purified and refolded scFv proteins. Genes for MLS128 scFv (S) and the long linker-version (L) that contained nine extra amino acids were constructed and subcloned into the pCI-Fc plasmid to create MLS128 scFv–Fc (S) and (L) pCI-plasmids. Both scFv–Fc pCI-plasmids were introduced into CHO-DHFR cells. MLS128 scFv–Fc (S) and (L) clones that survived in the selective medium were analysed for Fc positivity by assaying culture

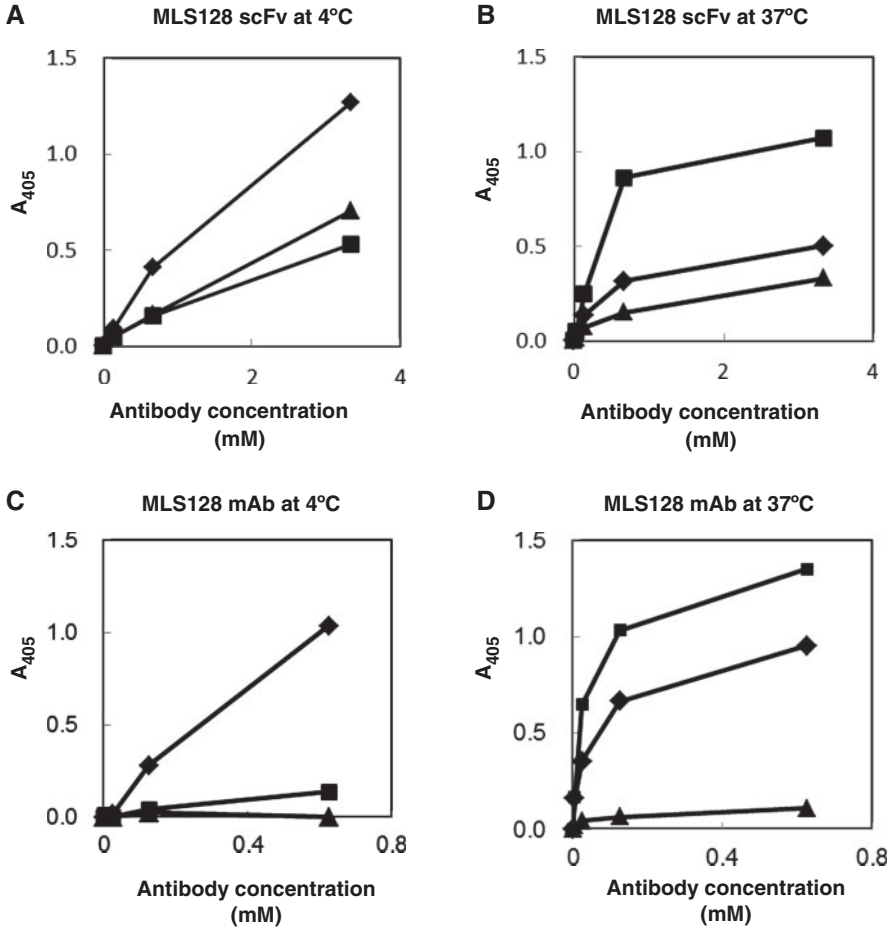

Fig. 4 Comparison of the Tn-antigen-binding activity of the purified and refolded MLS128 scFv protein and MLS128 mAb at 4 and 37°C. ELISA was carried out at both 4 and 37°C with the purified MLS128 scFv protein (A and B, respectively) as well as MLS128 as a control (C and D, respectively) as described in the Methods section. Immobilized in the wells were: filled diamond, Tn3-peptide; filled square, Tn2-peptide; filled triangle, BSA. C and D are adapted from (*31*) (Fig. 1A and B, respectively).

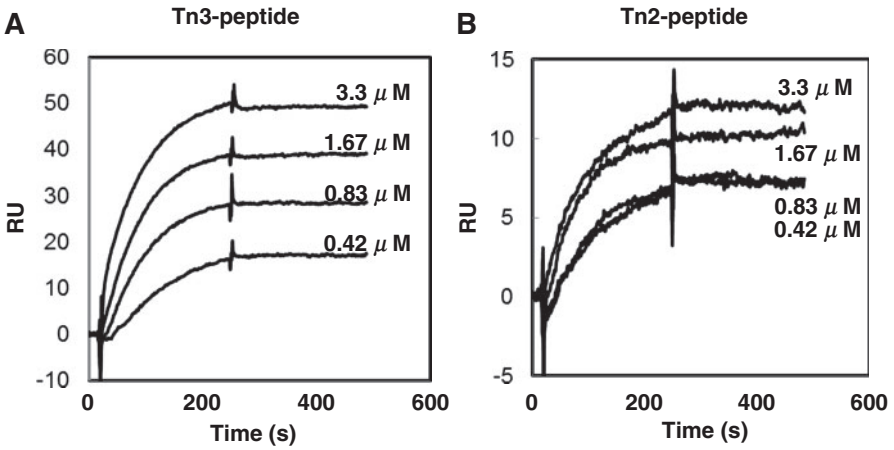

Fig. 5 Binding kinetics of purified MLS scFv protein according to SPR analyses at 10°C . Shown are sensorgrams of the binding of the MLS128 scFv protein at indicated concentrations to Tn3-peptide (A) and Tn2-peptide (B) immobilized on CM3 sensor chips.

supernatants with sandwich ELISA. Four positive clones were identified from both (date not shown). The clone that expressed the highest level of scFv–Fc was selected for large-scale production and purification of MLS128 scFv–Fc (S) or (L).

Starting with 145 and 170 ml of the media, 86 and 112 μg of MLS128 scFv–Fc (S) and (L) proteins, respectively, were purified by protein A-Sepharose column chromatography. The level of MLS128 scFv–Fc protein expression in the medium was thus

Table III. Kinetic parameters of MLS128 scFv (panel a) and mAbs (panel a and b) obtained from SPR.

	K_a (1/Ms)	K_d (1/s)	K_D (M)
Panel a: at 10°C (Fig. 5)			
Tn2-peptide	6.9×10^2	4.1×10^{-3}	5.9×10^{-6}
Tn3-peptide	5.0×10^3	5.5×10^{-3}	1.1×10^{-6}
Panel b: at 25°C [from (*15*)]			
Tn2-peptide	2.7×10^4	3.6×10^{-3}	1.3×10^{-7}
Tn3-peptide	4.5×10^4	1.1×10^{-3}	2.4×10^{-8}
Panel c: at 10°C (from (*31*))			
Tn2-peptide	2.2×10^3	2.9×10^{-4}	1.3×10^{-5}
Tn3-peptide	4.7×10^3	7.9×10^{-3}	1.7×10^{-6}

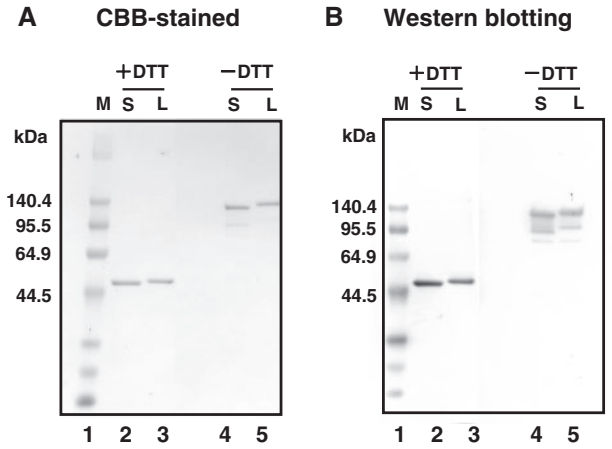

Fig. 6 SDS–PAGE and Western blotting of purified MLS128 scFv–Fc (S) and (L) proteins. Protein-A Sepharose-purified scFv–Fc proteins, 1.0 μg/lane and 0.6 μg/lane for CBB staining and Western blotting, respectively, were analysed on a 10% SDS–PAGE gel under reducing (lanes 2 and 3) and non-reducing (lanes 4 and 5) conditions. (A) Coomassie Brilliant Blue stained gel. (B) Western blotting with anti-human IgG antibody.

calculated to be 0.59 or 0.66 μg/ml for MLS128 scFv–Fc (S) and (L) proteins, respectively.

Purity and structural analyses of two types of purified MLS128 scFv–Fc proteins were examined using SDS–PAGE under reducing and non-reducing conditions followed by CBB-staining (Fig. 6A) or Western blotting with anti-human IgG antibody (Fig. 6B). Since human IgG_1 Fc contains a hinge region, the scFv–Fc produced by mammalian cells is expected to form a disulfide-linked dimer. The results clearly show that both MLS128 scFv–Fc proteins are dimeric, as is apparent in Fig. 6 lanes 2 and 3 and 4 and 5 for monomers and dimers, respectively. The molecular mass of the monomeric scFv–Fc estimated with SDS–PAGE under reducing conditions was ~52 kDa, which agrees with the theoretical molecular mass of 52.0 and 52.6 kDa for (S) and (L) types, respectively, based on their deduced amino acid sequences (Fig. 1). Differences in the sizes of MLS128 scFv–Fc (S) and (L) proteins were observed (Fig. 6, lanes 2 and 3). These results suggest that both long and short linker types of MLS128 scFv–Fc proteins were successfully produced and purified from CHO cells.

Assessments of the Binding Activity of Purified MLS128 scFv–Fc Proteins. The Tn3- and Tn2-peptides used in this study are well-defined epitopes for MLS128 that act as artificial ligands and are thus useful for determining affinity constants. However, glycoproteins such as asialo-agalaco-glycophorin A (GPA) and asialo-GPA were used as substrates to assess and compare the binding activity and specificity of two types of MLS128 scFv–Fc proteins. Thus, a natural glycoprotein, GPA, was selected as a ligand for affinity and specificity analyses of MLS128 scFv–Fc proteins. Since GPA is a highly glycosylated sialoglycoprotein with approximately 12 *O*-glycans, sequential treatment with sialidase and β-galactosidase results in removal of sialic acid and galactose residues, exposing T-antigen and Tn-antigen, respectively (*27*).

Figure 7 summarizes the binding activity of MLS128 scFv–Fc proteins and mAb as measured with ELISA using asialo- and asialo-agalacto-GPAs. Both MLS128 scFv–Fc (S) and (L) bound to asialo-agalacto-GPA (Tn-antigen-exposed GPA) in a concentration-dependent manner, but they did not bind to asialo-GPA (T-antigen-exposed GPA) at all, as shown in Fig. 7A and B, respectively. MLS128 scFv–Fc (S) exhibited greater binding activity than MLS128 scFv–Fc (L). Non-specific binding of MLS128 scFv–Fc proteins to agalacto-GPA or BSA was negligible, which is a good indication of successful refolding of the scFv–Fc proteins in CHO cells. This result is in a sharp contrast to a high level of non-specific binding observed with the MLS128 scFv proteins purified and refolded from inclusion bodies (Fig. 4). In summary, this experiment revealed that the activity ratios of scFv–Fc (S), scFv–Fc (L) and mAb to be approximately 3:1:72. This is consistent with the differences observed between the affinities of mAb and scFv protein since around one-order affinity reduction in recombinant scFvs from the parental mAb is generally observed (*32*).

Discussion

The major aim of this study was to isolate genes encoding MLS128 VH and VL to produce a large quantity of MLS128-binding domains such as scFv or scFv–Fc proteins. This in turn allows studies of the precise interactions between Tn3- or Tn2-epitopes and MLS128. MLS128 is the only known Tn3-specific mAb. MLS128 also has unique binding characteristics with regard to Tn3- and Tn2-ligands, and these two facts served as the impetus for this study. As described here, (i) genes encoding MLS128 variable domains were isolated; (ii) MLS128 scFv protein was expressed, purified and characterized; and (iii) MLS128 scFv–Fc (S) and (L) proteins were expressed, purified and characterized.

Genes encoding for VH and VL of MLS128 were cloned from hybridoma producing MLS128. The sequence homology of the VH domain to that of another anti-Tn-antigen mAb, 83D4, showed 96% amino acid sequence identity (Fig. 2). While 83D4 was shown to have similar affinities for both Tn2- and Tn3-peptides, MLS128 exhibited affinity for the

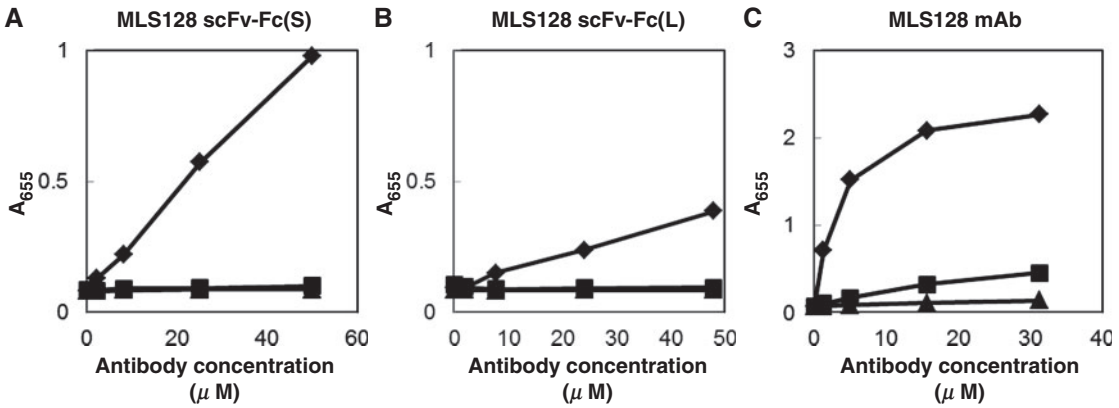

Fig. 7 Comparison of the binding activity of MLS128 scFv–Fc proteins and mAb as measured with ELISA using asialo- and asialo-agalacto-GPAs. Shown is the comparable binding activity of (A) MLS128 scFv–Fc (S), (B) MLS128 scFv–Fc (L), and (C) MLS128 mAb to asialo-agalacto-GPA (Tn-antigen-exposed GPA: filled diamond)., agalacto-GPA (T-antigen-exposed GPA: filled square) and BSA (filled triangle).

Tn3-peptide at a level similar to that of 83D4 but affinity an order lower for Tn2-peptide than that for Tn3-peptide (*15*; Table III, panel b). The VH domains of both mAbs, which share 96% homology, presumably contribute to Tn2/3-antigen-binding activity, but the difference in their VL domains may play an important role in determining the Tn3 specificity of MLS128. With respect to the VL domain of MLS128, 98% amino acid sequence identity was noted with anti-blood group A hybridoma A003-40/5G7k, which is IgM and specific for GalNAcα1-3(Fucα1-2)Galβ1 (blood group A) but does not bind to Galα1-3(Fucα1-2)Galβ1 (blood group B) (*29*, *30*). An interesting finding is that A003-40/5G7k specifically recognizes the GalNAcα-epitope that is the Tn-antigen motif. Understanding the contribution of the VL domain of MLS128 to Tn3-specificity may help to explain the different levels of specificity exhibited by MLS128 and 83D4.

Expression, purification and characterization of MLS128 scFv protein revealed that the purified and refolded scFv protein had the specificity and affinity characteristics of the parental MLS128 mAb. ELISA results (Fig. 4), however, indicated that the purified scFv proteins may have aggregated since a high level of non-specific binding was observed. As shown in Fig. 1, the scFv construct contained a short linker due to the primers used in this study. The short linker most likely caused dimerization of scFv monomers. In fact, dimers as well as large aggregates were detected by gel permeation chromatography (data not shown). SPR analyses of scFv proteins binding to Tn3- or Tn2-peptide, however, resulted in a range of affinity constants, as was expected.

The *E. coli* expression system is a cost-effective way to produce large quantities (gram scale) of recombinant protein for therapeutic and analytical uses. In this study, the pET expression system was thus used to express the MLS128 scFv gene. Although the expression vector contained the pelB leader sequence at the amino-terminus, which should allow accumulation of expressed proteins in the periplasm where the proteins should be properly folded, efforts to isolate scFv proteins from the periplasm were unsuccessful. Purification and refolding of proteins from inclusion bodies is not a desirable way to produce intact forms of recombinant antibodies, but optimized refolding conditions resulted in preparation of active MLS128 scFv proteins. The results described thus satisfied the goal of demonstrating the authenticity of MLS128 gene products.

MLS128 scFv–Fc (S) and (L) proteins were expressed, purified and characterized to further confirm the authenticity of the MLS128 gene products. As mentioned above, the original short linker may have caused the aggregation problem in the purified and refolded scFv proteins. Thus, an additional scFv–Fc construct containing a regular length linker, designated scFv–Fc (L), was constructed. Both MLS128 scFv–Fc (S) and (L) proteins were secreted into the media and successfully purified by protein A-Sepharose chromatography. The binding activity of the purified scFv–Fc (S) and (L) proteins to Tn-exposed GPA measured by ELISA using asialo-agalacto-GPA revealed that the ratio of scFv–Fc (S), scFv–Fc (L) and mAb (control) activity was approximately 3:1:72. The activity of scFv–Fc (L) was found to be lower than that of scFv–Fc (S). This suggests that subtle differences in the binding domains caused by the linkers are responsible for determining activity. Further investigation is necessary to explain the difference in activity observed with scFv–Fc (S) and (L). In short, the fact that expressed and purified MLS128 scFv–Fc proteins exhibited binding activity to Tn-antigen (asialo-agalacto-GPA) but not to T-antigen (agalacto-GPA) confirmed the authenticity of the cloned MLS128 genes.

In conclusion, this study has both laid the foundations for and also provided researchers with a tool for intensive analysis of Tn3- or Tn2-ligand and MLS128.

Accession numbers

MLS128 VH gene, Accession number AB663492 (DDBJ). MLS128 VL gene, Accession number AB663493 (DDBJ).

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Conflict of interest

None declared.

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