# Isolation and Characterization of Phage-Displayed Single Chain Antibodies Recognizing Nonreducing Terminal Mannose Residues. 2. Expression, Purification, and Characterization of Recombinant Single Chain Antibodies†

Wei Zhang,[‡,§,||] Ayano Matsumoto-Takasaki,[‡,§] Yu Kusada,[‡,§,||] Hiroyuki Sakaue,[§,||] Keiko Sakai,[‡,§] Munehiro Nakata,[§,||] and Yoko Fujita-Yamaguchi*,[‡,§,||]

*Institute of Glycotechnology, Tokai University, Kanagawa 259-1292, Japan Science and Technology Corporation Core Research Evolutional Science Technology, Japan, and Department of Applied Biochemistry, Tokai University School of Engineering, Kanagawa 259-1292, Japan*

*Received September 9, 2006; Revised Manuscript Received November 3, 2006*

ABSTRACT: Since phage-display technology is probably the best available strategy to produce antibodies directed against various carbohydrate moieties, we employed phage-display technology to generate human single chain antibodies (scFvs) using neoglycolipids as carbohydrate antigens. An accompanying paper in this issue describes how phage-displayed antibodies (phage Abs) that recognized nonreducing terminal mannose residues were isolated and characterized. In this study, four independent scFv genes, isolated by a mannotriose (Man3)-bearing lipid as an antigen as previously described, were used to construct expression vectors to produce soluble scFv proteins in quantity. Both bacterial and mammalian expression systems were used to produce glutathione S-transferase-scFv fusion proteins and scFv-human IgG1 Fc conjugates, respectively. The expressed scFv fusion proteins were purified to apparent homogeneity with yields of approximately 1 and 48 mg, from 1 L of bacterial culture and myeloma cell media, respectively. Surface plasmon resonance and ELISA analyses confirmed that purified scFv proteins showed Man3 specificity. The humanized antibody in scFv-Fc form, derived from clone 5A3, was a disulfide-liked dimer with a molecular mass of 108 kDa. According to a bivalent model, the kinetics parameters of its binding to Man3 were determined to be $k_a = 4.03 \times 10^4\ M^{-1}\ s^{-1}$, $k_d = 5.77 \times 10^{-4}\ s^{-1}$, $K_A = 6.98 \times 10^7\ M^{-1}$, and $K_D = 1.43 \times 10^{-8}$ M. This study thus established the foundation for isolation of carbohydrate-specific scFv genes and eventual production of humanized scFv-Fc type antibodies.

Because many carbohydrates are self-antigens by nature, immunization with carbohydrates often led to a primary IgM response and no response in some cases (*1*, *2*). Phage display technology is thus considered the best available strategy to produce antibodies directed against carbohydrate moieties. This technology has been used mostly to generate antibodies against proteins, whereas its use for carbohydrate antigens has been limited due to difficulties in immobilization of carbohydrate antigens alone onto plastic plates. Consequently, previous studies utilized glycoproteins, heteroglycans, and carbohydrate−bovine serum albumin (BSA)[1] conjugates as antigens to produce anti-carbohydrate single chain antibodies (scFvs) by phage display methods (*1*, *3*−*7*). In addition, most anti-carbohydrate antibodies reported thus far have relatively low affinity and are thus not suitable for in vivo diagnostics or therapy.

The short-term objectives of the current study were to establish a new methodology so that scFvs against desired carbohydrate moieties can be readily isolated and to produce soluble scFv proteins in quantity so that purification and characterization of isolated scFvs can be easily achieved. Two consecutive papers by the authors present the respective results of these objectives. A previous paper in this issue describes how 25 sequence-independent clones were isolated using a model neoglycolipid, mannotriose-dipalmitoylphosphatidylethanolamine (Man3-DPPE) and how phage antibodies as well as scFv protein preparations had good affinities and specificities for nonreducing terminal mannose residues. The present paper further describes characterization of the

† This work was supported by a grant from JST-CREST and in part by an NIH grant (CA65767).

* Corresponding author. Phone: +81-463-58-1211 (ex. 4188). Fax: +81-463-50-2012. E-mail: yamaguch@keyaki.cc.u-tokai.ac.jp.
‡ Institute of Glycotechnology, Tokai University.
§ JST CREST.
|| Department of Applied Biochemistry, Tokai University School of Engineering.

[1] Abbreviations: scFv, single-chain Fv; Man3, mannotriose [Manα1−6(Manα1−3)Man]; Man5, mannopentaose [Manα1−6(Manα1−3)-Manα1−6(Manα1−3)Man]; DPPE, dipalmitoylphosphatidylethanolamine; GST, glutathione *S*-transferase; IPTG, isopropyl *β*-D-thiogalactoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; OD, optical density; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; BSA, bovine serum albumin; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); SFM, serum-free medium; DAB, diaminobezidine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccinimide; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; LNT, lacto-*N*-tetraose (Gal*β*1−3GlcNAc*β*1−3Gal*β*1−4Glc); LNFPIII, lacto-*N*-fucopentaose III [Gal*β*1−4(Fucα1−3)GlcNAc*β*1−3Gal*β*1−4Glc]; LNnT, lacto-*N*-neotetraose (Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4Glc).

10.1021/bi0618767 CCC: $37.00 
Published on Web 01/02/2007

Table 1: Primers Used in PCR for Gene Cloning and Construction

| primers | sequences[a] |
|---|---|
| 5A3G1 | 5′-ATG*GGATCC*CAGGTGCAGCTGGCAG-3′ (*Bam*HI) |
| 5A3G2 | 5′-GAC*GAATTC*TAGGACGGTCAGCTTGGT-3′ (*Eco*RI) |
| 5C10G1 | 5′-ATG*GGATCC*CAGGTGCAGCTGGTGCAG-3′ (*BamHI*) |
| 5C10G2 | 5′-GAC*GAATTC*TTTAATCTCCAGTCGTGT-3′ (*EcoRI*) |
| F1 | 5′-CCCAAGCTT*GAATTC*CACCATGGAGACA-3′ (*Eco*RI) |
| R1 | 5′-CTGGATATCACCTGTGGAACC-3′ |
| F2 | 5′-ACAGGTGATATCCAGGTGCAGCTG-3′ |
| R2 (for 1A4-12) | 5′-CTAG*TCTAGA*TTTGATTTCCAC-3′ (*Xba*I) |
| R3 (for 1G4) | 5′-CTAG*TCTAGA*TTTAATCTCCAG-3′ (*Xba*I) |
| hFc1 | 5′-CTAG*TCTAGA*GATCCCAAATCTTGT-3′ (*Xba*I) |
| hFc2 | 5′-ATAGTTTA*GCGGCCGC*GTGTTTCATTTACCCGGAGA-3′ (*Not*I) |
| P1 | 5′-CCC*AAGCTT*GCCGCCACCATGGAGACA-3′ (*Hind*III) |
| P2 | 5′-C*TTAATTAA*T*GGCGCGCC*ACCTGTGGAACCTGGAAC-3′ (*Pac*I and *Asc*I) |
| P3 | 5′-*GGCGCGCC*A*TTAATTAA*GGACCCCAAATCTTCTGAC-3′ (*Pac*I and *Asc*I) |
| P4 | 5′-CCG*GAATTC*TCACTATTATTTCCCGGGAGACAGGGA-3′ (*Eco*RI) |

[a] Underlined and italic sequences are restriction sites for enzymes indicated in parenthesis.

isolated scFvs after establishing methods of production and purification.

In this study, four independent scFv genes, 1A4-12, 1G4, 5A3, and 5C10, isolated by Man3-DPPE as an antigen from human scFv-displayed phage library and whose characterization was described in the previous paper, were selected to construct expression vectors to produce soluble scFv proteins in quantity. Both bacterial and mammalian expression systems were used to produce and purify glutathione *S*-transferase (GST)-scFv fusion proteins and scFv-human IgG1 Fc conjugates, respectively. Surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) analyses confirmed that purified scFv proteins showed specificity to mannose residues. This and previous studies thus established the foundation for isolation of carbohydrate-specific scFv genes and eventual production of humanized scFv-Fc type antibodies.

## MATERIALS AND METHODS

*Materials.* Glutathione Sepharose 4B beads for purification of GST and its fusion proteins were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). The cDNA clone of human IgG1 Fc, composed of hinge, CH2 and CH3, was originally from Dr. J. Schlom, Laboratory of Tumor Immunology and Biology, Division of Cancer Biology and Diagnosis, NCI (Bethesda, MD). Mock plasmid pEE12.4 and murine myeloma NS0 cell strain were obtained from Lonza Biologics (Slough, UK), and the vector pCI-neo and Wizard *Plus* Midipreps (Cat.#A7640) were purchased from Promega (Madison, WI). Dulbecco's modified Eagle medium (DMEM, high glucose) was purchased from Invitrogen (Paisley, UK). Goat anti-human IgG (Fc fragment specific) and peroxidase-conjugated rabbit anti-human IgG secondary antibody were from Jackson Immunoresearch Inc. (West Grove, PA). Serum-free medium (SFM) used for CHO-DHFR$^-$ (HyQSFM4CHO) and NS0 (HyQSFM4Mab) were obtained from Hyclone (South Logan, UT). A DAB detection kit was purchased from Invitrogen. Protein A cartridge (Cat. No. 732-0091) was obtained from Bio-Rad (Hercules, CA). Mannose-BSA, Glucose-BSA, Man3-BSA and LNFPIII-BSA were obtained from Dextra Laboratories (Reading, UK). Galactose-sp-biotin, Man3-sp-biotin, LNnT-sp-biotin, LNFPIII-sp-biotin, and BSA-sp-biotin were from GlycoTech (Gaithersburg, MD).

*Bacterial Strains and Transformation. Escherichia coli* strain JM109 was used as a host for all plasmid preparations except for *E. coli* BL21 (DE3) that was used for the expression of GST-scFv fusion proteins. Plasmid transformations were performed by electroporation using the Gene Pulser II Apparatus (Bio-Rad) under conditions of capacitance at 25 $\mu$F, a resistor at 200 ohms (pulse controller), and voltage at 1.5 kV. Resulting bacteria were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2) containing ampicillin (100 mg/L).

*Expression and Purification of GST-scFv Fusion Proteins.* The 5A3 and 5C10 scFv genes amplified from phagemids encoding respective scFv genes as templates using primers 5A3-G1 and -G2 and 5C10-G1 and -G2, respectively (Table 1), was ligated into *Bam*HI-*Eco*RI-digested pGEX-4T-1-5A3 or 5C10 scFv plasmid (downstream of the GST gene) to generate an expression vector for a GST-fused scFv protein (GST-scFv). The recombinant expression plasmid, pGEX-4T-1-scFv (5A3 or 5C10), was prepared in JM109 and electroporated into *E. coli* BL21(DE3). The sequence of pGEX-4T-1-scFv (5A3 or 5C10) was confirmed by DNA sequencing. For the production of soluble GST-fused scFv proteins, recombinant *E. coli* cells from a seed culture were transferred to fresh LB medium containing ampicillin and cultured at 37 °C with shaking. After the optical density (OD600) of the culture reached about 0.3, different concentrations of isopropyl $\beta$-D-thiogalactoside (IPTG) were added to induce gene expression. After an additional 3.5 h of culturing at 20 °C with shaking at 200 rpm, bacterial cells were collected by centrifugation at 8000*g* for 30 min. Cell pellets were resuspended in phosphate-buffered saline (PBS) and disrupted on ice with a sonicator. The cell homogenate was centrifuged at 12000*g* for 15 min, and the supernatants containing GST fusion proteins were collected. The fusion proteins were purified by Glutathione Sepharose 4B beads according to the manufacturer's instructions. The eluates were extensively dialyzed against PBS. Samples were analyzed by SDS−PAGE, followed by staining with Coomassie Brilliant Blue (CBB) R-250.

*Design and Construction of Recombinant Vectors of Signal-scFv-human Fc.* Two mammalian systems, neo/CHO and GS/NS0, were used for expression of scFv conjugated with the human IgG1 Fc domain. The construct included a consensus ribosome-binding sequence and the signal peptide

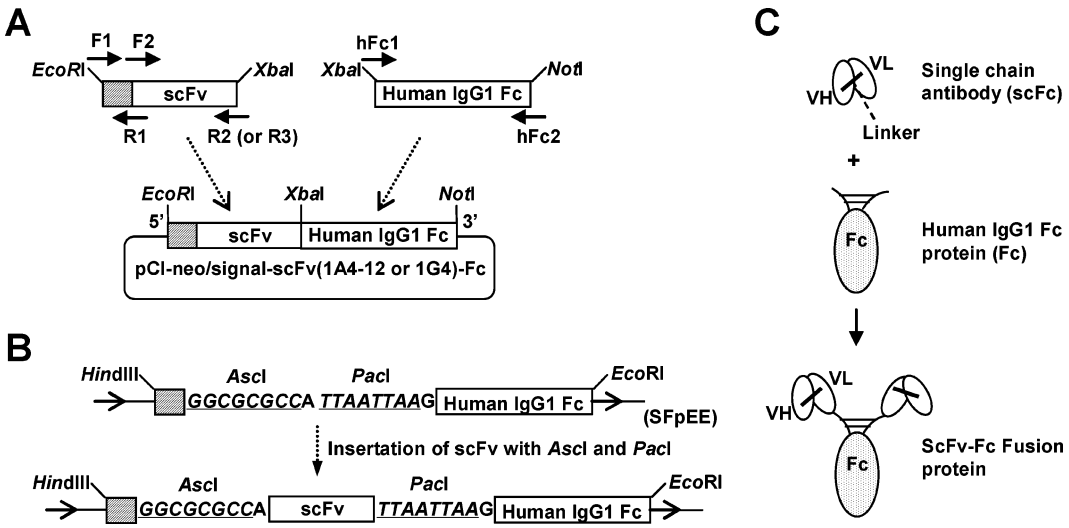


FIGURE 1: Construction of recombinant vectors of signal-scFv-human Fc. (A) Assembly of the gene encoding signal-scFv of 1A4-12 or 1G4 by splice-overlap extension PCR with human Fc in pCI-neo plasmid. The genes of signal-scFv and human Fc were inserted into the vector successively to construct the recombinant plasmid. Hatched box denotes the signal sequence. (B) The construction of the template plasmid signal-human Fc-pEE12.4 (SFpEE) for all scFvs insertation and expression in pEE12.4 plasmid. Between signal peptide and human IgG1 Fc tail, *Asc*I and *Pac*I sites were introduced for scFv insertion. For correct codon of amino acids of scFv and human Fc after introducing the above two enzyme sites, one additional neucleotide A was introduced after *Asc*I site and G after *Pac*I. Fusion of the gene segment was introduced into pEE12.4 by *Hin*dIII and *Eco*RI. ScFv genes were inserted by sites of *Asc*I and *Pac*I. (C) Schematic diagram of expected human Fc alone expressed by plasmid SFpEE and scFv-Fc fusion proteins expressed by recombinant plasmids after insertion of scFv genes.

from a murine kappa light chain previously used for high level mammalian antibody secretion (*8*, *9*). The signal peptide was followed by scFv and the human IgG1 Fc domain (see Materials).

Figure 1A summarizes the assembly of the gene encoding 1A4-12 or 1G4 scFv with the signal peptide sequence and human Fc domain by splice-overlap extension PCR followed by subcloning into pCI-neo plasmid resulting in pCI-neo/signal-scFv(1A4-12 or 1G4)-Fc. Primers used for PCR are listed in Table 1.

To easily insert a variety of scFv fragments into pEE12.4 vector for cloning and expression of scFv-Fc proteins, a template plasmid Signal-human Fc-pEE12.4 (SFpEE) was constructed. *Asc*I and *Pac*I sites were introduced into recombinant SFpEE between signal and human IgG1 Fc fragment for the insertion of a scFv gene. For the correct codon of the amino acid after introduction of the above two enzyme sites, an additional nucleotide A was introduced after the *Asc*I site and nucleotide G after *Pac*I. Figure 1B summarizes the assembly of a signal peptide with the human Fc domain as well as the insertion sites for scFv. The gene segment was produced by splice overlap extension PCR using the following primers and then introduced into pEE12.4 linearized by *Hin*dIII and *Eco*RI. Briefly, primers *P*1 and *P*2 (Table 1) were used to amplify the signal peptide (60 bp). Primers *P*3 and *P*4 (Table 1) were used to amplify human IgG1 Fc fragments (696 bp). Primers *P*2 and *P*3 contained overlap sequences. After purification by agarose gel electrophoresis, the two PCR products of signal peptides and human IgG1 Fc were mixed and subjected to PCR for a couple of cycles. Then primers *P*1 and *P*4 were added to produce the fusion gene. All plasmids used for electroporation into mammalian cells were purified by Wizard *Plus* Midipreps (Cat. No. A7640) and stored in sterile water.

*Cell Culture, Transfection and Screening.* Chinese hamster ovary (CHO-DHFR$^-$, ATCC) cells were cultured in DMEM (high glucose) with $10^{-4}$ M hypoxanthine, $10^{-5}$ M thymidine, 10% fetal bovine serum (FBS), and 2 mM glutamine. G418 at a concentration of 500 $\mu$g/mL was added to the above medium for selection of positive CHO-DHFR$^-$ clones. The murine myeloma NS0 cell line was grown in DMEM supplemented with 10% FBS and 2 mM glutamine. Selective medium for human Fc-expressing NS0 cells consisted of glutamine-free DMEM, dialyzed FBS, and glutamine synthase supplement (JRH Biosciences, Lenexa, KS).

In a 0.4 cm cuvette, 10 $\mu$g of linearized DNA was transfected into $2 \times 10^5$ CHO cells in PBS with a Bio-Rad Gene Pulser set to 0.3 kV and 950 $\mu$F. On day 3, cells were collected, counted and placed in 96-well plates ($\sim$50 cells/well) in the selective medium containing 500 $\mu$g/mL G418. The wells were changed with fresh media every 3 days. Forty micrograms of linearized DNA in sterile water was electroporated into $1 \times 10^7$ NS0 cells at conditions of 0.25 kV and 400 $\mu$F capacitance. Cells at $1.6 \times 10^5$ cell/mL were plated in 96-well plates, 50 $\mu$L/well, in nonselective media. Twenty-four hours post-electroporation, 150 $\mu$L of selective medium was added to each well, and cells were allowed to recover and grow undisturbed for about 3 weeks until discrete surviving colonies appeared.

Supernatants of transformed cells that were able to grow under selective conditions were screened for scFv-Fc protein secretion by sandwich ELISA. Goat anti-human IgG (Fc fragment specific) was diluted in 0.05 M bicarbonate coating buffer, pH 9.6, to a final concentration of 10 $\mu$g/mL and coated onto 96-well plates, 100 $\mu$L/well, at 4 °C overnight. After blocking with 3% BSA in PBS, cell culture supernatants from different clones were added and incubated at 37 °C for 1 h. After the samples were washed with PBS, horse radish peroxidase (HRP)-conjugated anti-human IgG antibody diluted to 1:20000 was added to the wells. The wells were washed with PBS and then incubated with 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The color

produced in the wells of plates was analyzed spectrophotometrically at 415 nm on a microplate reader (Bio-Rad model 680).

*Purification of scFv-Fc Antibody from SFM Media.* The best clone (4C2) for 5A3 scFv-Fc, which had the highest expression of antibody both in selective medium and in SFM (HyQSFM4Mab), was cultured on a large scale in a T175 flask in selective medium. When the cells reached the logarithmic phase of growth, the selective medium was replaced with SFM. After culturing in SFM for 24-48h, cell culture supernatants were collected and adjusted to pH 8.0 by adding 1/20 volume 1.0 M Tris (pH 8.0) and then passed through a protein A column (5 mL). The column was washed with 10 column volumes of 100 mM Tris-HCl buffer, pH 8.0. ScFv-Fc was eluted from the column with 100 mM citrate buffer pH 3.0 and collected in 1.5-mL conical tubes containing 1/10 volume 1 M Tris-HCl, pH 8.0. The best clone producing only the human Fc domain was screened and then subjected to the same purification procedures as above. The purified samples were dialyzed against PBS and analyzed by SDS−PAGE. For Western blot analyses, samples were transferred onto a PVDF membrane (GE Healthcare Bio-Sciences) and the scFv-Fc or Fc protein was visualized by reaction with rabbit-anti human IgG antibody conjugated with peroxidase using the DAB detection kit. Protein concentration was measured according to the procedures used by Bradford with BSA as a standard.

*Gel Filtration.* GST-scFv and scFv-Fc fusion proteins obtained as described above were fractionized by gel filtration using a Superdex 200 column (10 × 300 mm; GE Healthcare Bio-Sciences) equilibrated with 10 mM HEPES, pH 7.4, containing 135 mM NaCl, 10 mM $CaCl_2$ and 0.005% Tween 20 (a running buffer for SPR analyses) at a flow rate of 0.4 mL/min. Elution was monitored at 280 nm. Fractions (0.5 mL each) collected were subjected to SPR analyses as described below.

*SPR Analyses.* Real-time measurements for the binding of 5A3 scFv fusion proteins, GST-scFv and scFv-Fc, to the antigen, Man3, were evaluated using SPR. All SPR analyses were performed at 25 °C on a Biacore3000 biosensor (Biacore, Inc., Uppsala, Sweden). Man3 conjugated with BSA (Man3-BSA) and BSA as a control were immobilized onto CM3 sensor chips using the amine coupling kit supplied by the manufacturer. Binding of scFv fusion proteins to carbohydrate moieties on the surface of the sensor chips was monitored at a flow rate of 30 μL/min in a running buffer at a flow rate of 30 μL/min. The rates of association ($k_a$) and dissociation ($k_d$) for GST-5A3 scFv and 5A3 scFv-Fc were determined using BIAevaluation version 3.0 software (Biacore) by a bivalent analyte model (*10*), and the thermodynamic dissociation constant ($K_D$) was derived by dividing $k_d$ by $k_a$.

*ELISA.* Solid-phase ELISA was used to evaluate the antigen-binding activity of purified 5A3 scFv-Fc aginst Man3-BSA. Briefly, the antigen, Man3-BSA was diluted in 0.05 M bicarbonate coating buffer, pH 9.6, and coated onto 96-well ELISA plates with 10 μg/mL, 50 μL/well at 4 °C overnight with triplicate samples. After blocking of the sample with 3% BSA/PBS, a series of diluted samples of 5A3 scFv-Fc were added. After 2 h of incubation at room temperature, the bound scFv-Fc was detected with rabbit peroxidase-conjugated anti-human IgG antibody (1:20000 dilution) using ABTS as a substrate. After 30 min of incubation in the dark, the reaction was terminated by adding 100 μL of 2% oxalic acid solution. Absorbance at 415 nm was measured using a plate reader. To evaluate the specificity of 5A3 scFv-Fc, a group of different kinds of carbohydrate antigens conjugated with either BSA (0.5 μg/well) or biotin (50 pmol/well) were coated onto the ELISA plates, and the assays were carried out as described above.

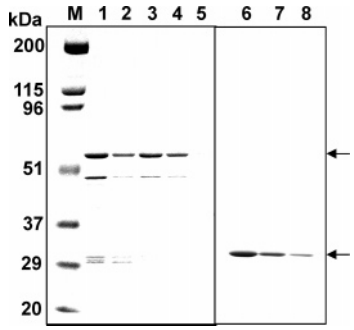


FIGURE 2: SDS−PAGE analysis of the purified GST-5A3 scFv fusion protein and GST alone. Lanes 1−5: the purified fusion proteins of GST-5A3 scFv (55 kDa) induced at 20 °C when the IPTG concentration was 0.2 (lane 1), 0.1 (lane 2), 0.05 (lane 3), 0.01 (lane 4), and 0 mM (lane 5). Lanes 6−8: purified GST (29 kDa) of 2 (lane 6), 1 (lane 7), and 0.5 μg (lane 8) from mock plasmid, pGEX-4T-1, induced at 20 °C when the IPTG concentration was 0.1 mM. M denotes the molecular weight marker. Proteins in the gels were stained with CBB. Upper and lower arrows indicate the GST-scFv and GST, respectively.

## RESULTS

*Expression and Purification of GST-scFv Fusion Protein.* *E. coli* BL21(DE3) cells, into which the recombinant expression plasmid, pGEX-4T-1-scFv (5A3 or 5C10), was introduced, were subjected to induction of soluble GST-5A3 or -5C10 scFv fusion protein. After disruption of cell pellets with a sonicator, the supernatants containing fusion proteins were purified by Glutathione Sepharose 4B beads. As the control, the mock plasmid pGEX-4T-1, which should express the GST protein alone at ∼29 kDa, was subjected to the same procedures of expression and purification as for the fusion protein. Figure 2 shows that the GST-5A3 scFv fusion protein with ∼55 kDa was expressed in a soluble form after induction by IPTG at concentrations of 0.01−0.2 mM at 20 °C since fusion proteins appeared to form inclusion bodies at 25, 30, or 37 °C (data not shown). Although purified GST-5A3 scFv protein preparations contained some contaminants, they were pure enough for the following SPR analyses. Yields for the GST-5A3 and 5C10 scFv fusion proteins and GST protein alone were 1.2, 1.0, and 3.8 mg from a 1-L culture, respectively.

*Production and Purification of scFv-Fc from Serum-Free Medium.* For production of scFv-Fc proteins, the human IgG1 Fc domain was constructed and first introduced into the neo/CHO system (pCI-neo vector from Promega, CHO-DHFR$^-$ cell strain from ATCC). Although the plasmid constructs for production of two anti-Man3 clones, 1A4-12 and 1G4, were introduced into this system, only a few and weak positive clones were recovered (Table 2). Even after large-scale culturing in SFM, neither of the two scFv-Fc proteins was purified (data not shown), which indicated that 1A4-12 and 1G4 scFv-Fc proteins were not produced in the neo/CHO

Table 2: Production and Purification of scFv-Fc Proteins in Two Mammalian Systems

| cell line | scFv-Fc protein | number of clones analyzed by ELISA | Fc-positive[a] | expression levels in SFM ($\mu$g/mL)[b] |
|---|---|---|---|---|
| CHO | 1A4-12 | 48 | 5 | 0 |
| | 1G4 | 36 | 2 | 0 |
| NS0 | 1A4-12 | 98 | 0 | ND[c] |
| | 1G4 | 80 | 0 | ND |
| | 5A3 | 25 | 6 | 48 |
| | Fc alone[d] | 12 | 10 | 120 |

[a] Expression of various scFv-Fc and control Fc proteins in two mammalian systems was examined by sandwich ELISA using anti-human IgG (Fc fragment specific) antibody. [b] Expression levels of scFv-Fc proteins in SFM were estimated from the yields of the proteins after purification by protein A column chromatography. [c] Not determined. [d] Fc domain only was expressed as a control.

system. Alternatively, the GS/NS0 system as used previously (*11*, *12*) was utilized for expression and production of scFv-Fc proteins. Of 1A4-12, 1G4 and 5A3 scFv-Fc genes constructed in the pEE12.4 plasmid and introduced into NS0 cells, the 5A3 scFv-Fc gene was the only clone that displayed good expression in the GS/NS0 system. Of 25 stable clones grown in selective media, six were found to be Fc-positive in media by ELISA (data not shown). Among the clones examined, the 4C2 clone exhibited the highest level of 5A3 scFv-Fc protein production in both serum-containing and serum-free media (data not shown). The scFv-Fc protein produced by the GS/NS0 system in serum-free media was purified by protein A-column chromatography. A total of 4.3 mg of 5A3 scFv-Fc protein was purified from 90 mL SFM of 4C2 clone. The level of 5A3 scFv-Fc protein expression in the medium was thus calculated to be approximately 48 $\mu$g/mL for the 4C2 clone (Table 2). The purified scFv-Fc protein was used for binding activity analyses by ELISA and SPR. In contrast to 5A3, 1A4-12 and 1G4 scFv-Fc proteins were still not expressed in the GS/NS0 system since no Fc-positive clone was detected among 98 and 80 clones examined by ELISA, respectively (Table 2). The human IgG1 Fc domain alone, as a control to validate the GS/NS0 expression system, was expressed and purified simultaneously. The clone with the human IgG1 Fc gene introduced expressed the Fc protein at a considerably high level (120 $\mu$g/mL) in SFM (Table 2). Since it is possible that anti-Man3 scFv-Fc proteins may be trapped in endoplasmic reticulum where high mannose-type oligosaccharides are abundant, myeloma cell lysates of above-mentioned clones were subjected to SDS−PAGE and immunoblotting with anti-Fc antibody. The results indicated that only the cell lysates of 5A3 and Fc clones contained scFv-Fc and Fc proteins, respectively, whereas 1A4-12 and 1G4 scFv-Fc genes-introduced NS0 cells did not show any Fc-reactivity (data not shown).

*Purity and Structural Analysis of Purified 5A3 scFv-Fc Protein.* The purified 5A3 scFv-Fc protein was analyzed by SDS−PAGE and immunoblotting under nonreducing and reducing conditions (Figure 3). Since human IgG1 Fc contains a hinge region, the scFv-Fc produced by mammalian cells is expected to form a disulfide-linked dimer. Results clearly shows that the 5A3 scFv-Fc protein is a dimer consisting of two monomers. The molecular mass of the monomeric scFv-Fc estimated by SDS−PAGE and immunoblotting was 53−55 kDa, which is consistent with that calculated from the amino acid sequence, 54 098 (Figure 3). The scFv-Fc is thus a divalent antibody that contains the human IgG1 Fc domain and has an expected molecular mass of 108 kDa. The molecular mass of the dimeric scFv-Fc under nonreducing conditions estimated by SDS−PAGE and immunoblotting was not precisely double that of the monomer under reducing conditions. This is possibly due to the post-translational modification with *N*-glycation of the Fc region as observed with regular antibodies and/or a structural effects during electrophoresis (*13*). Therefore, it was concluded that the S−S bonds of scFv-Fc were correctly constructed as expected from the sequence.

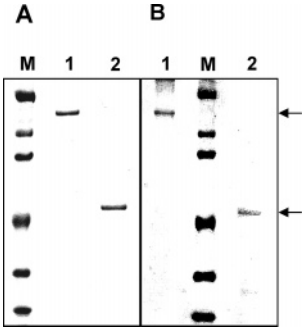


FIGURE 3: SDS−PAGE and immunoblotting of Protein A affinity-purified 5A3 scFv-Fc protein. (A) Coomassie staining on 4−20% acrylamide gel. (B) Immunoblotting with HRP labeled anti-human IgG (Fc specific) antibody. Lane 1, purified 5A3 scFv-Fc under nonreducing conditions; Lane 2, purified 5A3 scFv-Fc under reducing conditions; M, molecular weight maker (201, 115, 96, 52, 38, and 29 kDa).

*SPR Analyses of 5A3 scFv Proteins against Man3 Antigen.* SPR studies were performed to assess the ability of the anti-Man3-scFv to bind to the target antigen Man3, in two recombinant soluble forms, GST-scFv and scFv-Fc proteins, expressed in *E. coli* and mammalian systems, respectively.

Initial binding studies performed with the purified GST-5A3 scFv protein indicated that it had binding specificity to Man3-BSA over LNFPIII-BSA, whereas the GST protein alone did not show binding activity to either carbohydrate moieties (data not shown). Another anti-Man3 clone, 5C10, similarly expressed and purified as a GST fusion protein, also showed binding activity to Man3 (data not shown). Kinetic parameters of the purified GST-5A3 scFv protein to Man3-BSA were determined by SPR measurements at five concentrations with the range of 46−295 nM (Figure 4). The sensorgrams shown in Figure 4A were fitted to a bivalent analyte model since it was found that the GST-5A3 scFc as a fusion protein as well as the GST protein formed dimmers under the conditions used as determined by Superdex 200 gel filtration (data not shown). The kinetics parameters as determined by BIAevaluation software were $k_a = 3.08 \times 10^3$ M$^{-1}$ s$^{-1}$, $k_d = 6.71 \times 10^{-2}$ s$^{-1}$, $K_A = 4.59 \times 10^4$ M$^{-1}$, and $K_D = 2.18 \times 10^{-5}$ M.

Further SPR studies were performed with the divalent scFv-Fc proteins purified as monomers by gel filtration. The 5A3 scFv-Fc protein solutions at four different concentrations in the range of 6.7−26.8 nM were passed over the immobilized Man3-BSA at a flow rate of 30 $\mu$L/min. The sensorgrams shown in Figure 4B were fitted to a bivalent model. The kinetics parameters as determined by BIAevaluation software were $k_a = 4.03 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_d = 5.77 \times$

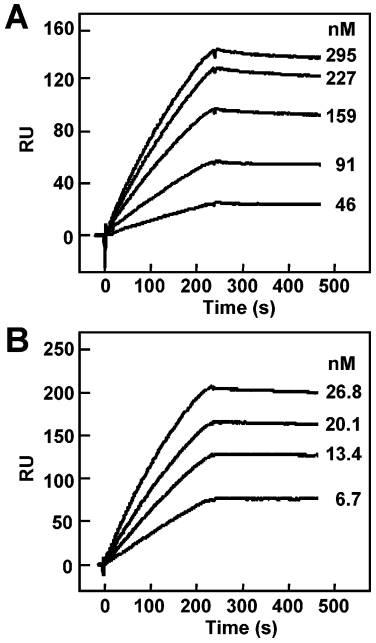


FIGURE 4: Binding kinetics curves of 5A3 scFv fusion proteins with immobilized Man3 antigen by SPR analyses. (A) Sensorgrams of the binding of GST-5A3 scFv to the immobilized Man3-BSA. The purified GST-5A3 scFv proteins were injected at the indicated concentrations. (B) Sensorgrams of the binding of 5A3 scFv-Fc to the immobilized Man3-BSA. The purified 5A3 scFv-Fc proteins were injected at the indicated concentrations. The binding curves were derived from one particular experiment where the different concentrations of the fusion proteins were analyzed on the same Man3-BSA-immobilized CM3 sensor chip.

Table 3: Kinetic Parameters of 5A3 Fusion Proteins Binding to Man3-BSA[a]

| fusion proteins | $k_a$ ($M^{-1}s^{-1}$) | $k_d$ ($s^{-1}$) | $K_A$ ($M^{-1}$) | $K_D$ (M) |
|---|---|---|---|---|
| GST-5A3 scFv | $3.08 \times 10^3$ | $6.71 \times 10^{-2}$ | $4.59 \times 10^4$ | $2.18 \times 10^{-5}$ |
| 5A3 scFv-Fc | $4.03 \times 10^4$ | $5.77 \times 10^{-4}$ | $6.98 \times 10^7$ | $1.43 \times 10^{-8}$ |

[a] Kinetic parameters $k_a$ and $k_d$ were measured by SPR, and $K_A$ and $K_D$ were calculated as $k_a/k_d$ and $k_d/k_a$, respectively.

$10^{-4}$ $s^{-1}$, $K_A = 6.98 \times 10^7$ $M^{-1}$, and $K_D = 1.43 \times 10^{-8}$ M. Table 3 summarizes the results of kinetic parameters of two 5A3 fusion proteins to Man3-BSA. SPR analyses were also carried out in the presence of 50 mM α-methyl mannoside, which inhibited the binding of the purified GST-5A3 and scFv-Fc proteins at 295 and 26.8 nM, respectively (data not shown). These results strongly indicated that the purified fusion scFv proteins bind to the Man3 moiety.

*Binding Specificity of 5A3 scFv-Fc by ELISA.* The binding of the 5A3 scFv-Fc protein to Man3-BSA coated onto plastic plates (0.5 μg/well) as a function of scFv-Fc concentration is shown in Figure 5A. The best concentration of 5A3 scFv-Fc protein for detection of binding activity with respect to Man3 was 20 μg/mL, which was used for ELISA analyses with a group of different kinds of carbohydrate-antigens conjugated to BSA (Figure 5B) or biotin (Figure 5C). The results of analyses for antibody specificity suggest that the purified 5A3 scFv-Fc possesses the ability to specifically recognize Man3.

## DISCUSSION

The objective of previous and present studies was to establish a new methodology by which scFvs against desired carbohydrate moieties can be isolated and produced as soluble scFv proteins in quantity. This research's long-term objective is to develop such scFvs for application in cancer therapeutics such as tissue-specific delivery of anticancer drugs as well as in producing antibody-based anti-cancer therapeutics and in diagnostics for catastrophic diseases including pancreatic cancer for which early detection is currently not available.

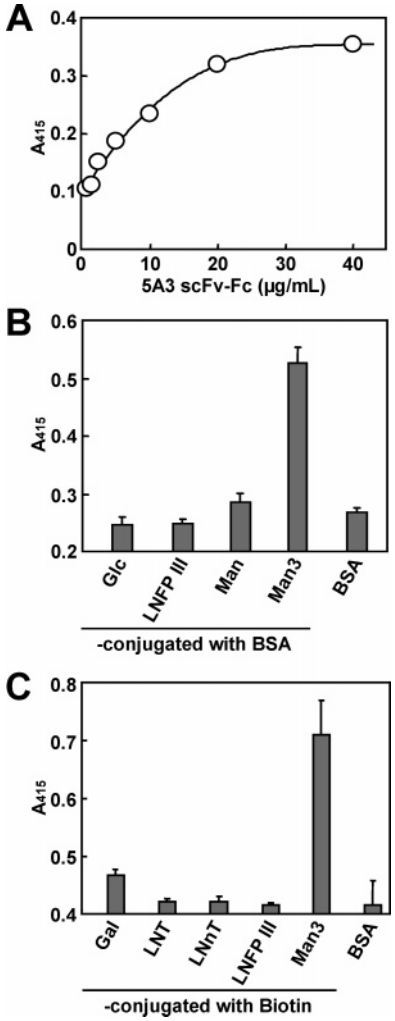


FIGURE 5: Antigen special recognition of 5A3 scFv-Fc by ELISA. Binding of a series of diluted samples of purified 5A3 scFv-Fc to Man3-BSA (0.5 μg/well) (A). Binding of the same concentration of 5A3 scFv-Fc to a group of different carbohydrate antigens conjugated with BSA (B) or biotin (C). BSA conjugates (0.5 μg/well) or biotin conjugates (50 pmol/well) were absorbed onto the surface of wells. The purified scFv-Fc samples were applied to the wells with the same concentration of 20 μg/mL and then detected with HRP-conjugated anti-human IgG (Fc specific) secondary antibody.

A previous paper showed that 25 sequence-independent clones were isolated using a neoglycolipid, Man3-DPPE, a model carbohydrate antigen and that phage antibodies as well as scFv protein preparations had good affinities and specificities for nonreducing terminal mannose residues. Also shown were complete amino acid sequences of 15 independent clones. As is readily apparent, the most valuable aspect of phage display technology is that gene profiling and gene manipulation can readily be achieved. The present paper described expression, production, and purification of the previously isolated scFvs.

Thus far, four independent anti Man3-scFv genes, 1A4-12, 1G4, 5A3, and 5C10, have been used to construct expression vectors to produce soluble scFv proteins in quantity. A bacterial expression system was first used to produce 5A3 and 5C10 GST-scFv fusion proteins since mammalian expression systems are time-consuming and labor-intensive. Although only the results for 5A3 are presented in this paper, both fusion proteins were expressed and purified. The GST fusion proteins, however, did not appear to be abundantly expressed as expected. In addition, this work sought to construct humanized antibodies with an eye toward developing antibody therapeutics. Thus, efforts shifted to the mammalian expression system. Of the two mammalian expression systems, the neo/CHO system was originally tested. As summarized in Table 2, positive clones producing scFv-Fc proteins were not obtained from either the 1A4-12 or 1G4 scFv gene construct, although five and two clones, respectively, grew in selective medium. These two genes were subcloned into pEE12.4 and introduced into NS0 cells. No clone grew, however, in selective medium (Table 2). In contrast, the 5A3 scFv-Fc protein was successfully produced in the GS/NS0 system. The expression level of 5A3 scFv-Fc protein was 48 $\mu$g/mL, which was significantly lower than 120 $\mu$g/mL as obtained when the Fc domain alone was expressed. When compared to the bacterial expression of GST-scFv fusion protein, however, this is considered to be highly productive as evidenced by the difference in the yields of approximately 1 mg from 1 L of bacterial culture versus 48 mg from 1 L of myeloma cell media. These results suggest that the GS/NS0 system is suitable because a good yield was achieved and because it allowed humanized antibody production through introduction of human IgG1 Fc.

It should be noted that expression of anti-Man3 scFv-Fc proteins in myeloma cells was extremely difficult. As described above, both 1A4-12 and 1G4 scFv-Fc proteins were never recovered in two mammalian expression systems used. Of clones grown in the neo/CHO system, Fc-positive clones were 10% (5/48) and 5.6% (2/36) for 1A4-12 and 1G4, respectively, whereas no Fc-positive clone were detected in the GS/NS0 system (Table 2). Even though 5A3 scFv-Fc positive clones were grown in NS0 cells, which eventually led to successful purification, only 24% (6/25) of clones grown in selective medium produced scFv-Fc. This is a sharp contrast to the number of clones producing only the Fc domain (83% of clones tested were positive) (Table 2). Anti-Lewis x scFv-Fc protein was also produced in the GS/NS0 system. Of 97 clones tested, 78% showed production of scFv-Fc in medium (manuscript in preparation). These observations suggest that anti-Man3 scFv-Fc proteins may be harmful to NS0 cells. It is thus most likely that NS0 cells expelled the anti-Man3 scFv-Fc gene to survive, resulting in few clones appearing in selective media. Thus, being able to produce the 5A3 scFv-Fc protein in mammalian cells was highly fortunate. A plausible conclusion is that the specificity and affinity of this particular anti-Man3 scFv must have helped it avoid harming the host cell.

SPR and ELISA analyses confirmed that the purified 5A3 scFv-Fc protein showed Man3 specificity with an affinity constant of $K_D = 1.43 \times 10^{-8}$ M. This and previous studies thus established isolation of carbohydrate-specific scFv genes and eventual production of humanized scFv-Fc type antibodies. The affinity constant of $10^{-8}$ is high for an antibody against carbohydrates. This indicates that isolated scFvs may be used as-is to construct antibody therapeutics such as multimeric antibodies with different specificities or to improve its original affinity by affinity maturation procedures. In summary, this study has provided the foundation for development of carbohydrate-specific antibody-based therapeutics and diagnostics.

## REFERENCES


1. Deng, S., MacKenzie, C. R., Sadowaka, J., Michniewicz, J., Young, N. M., Bundle, D. R., and Narang, S. A. (1994) Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display, *J. Biol. Chem. 269*, 9533−9538.
2. MacKenzie, C. R., Hirama. T., Deng, S., Bundle, D. R., Narang, S. A., and Young, N. M. (1996) Analysis by surface plasmon resonance of the influence of valence on the ligand binding affinity and kinetics of an anti-carbohydrate antibod, *J. Biol. Chem. 271*, 1527−1533.
3. Babino, A., Pritsch, O., Oppezzo, P., Du Pasquier, R., Roseto, A., Osinaga, E., and Alzari, P. M. (1997) Molecular cloning of a monoclonal anti-tumor antibody specific for the Tn antigen and expression of an active single-chain Fv fragment, *Hybridoma 16*, 317−324.
4. van Kuppevelt, T. H., Dennissen, M. A. B. A., van Venrooij, W. J., Hoet, R. M. A., and Veerkamp, J. H. (1998) Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney, *J. Biol. Chem. 273*, 12960−12966.
5. Mao, S., Gao, C., Lo, C.-H. L., Wirsching, P., Wong, C.-H., and Janda, K. D. (1999) Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewis x and Lewis x, *Proc. Natl. Acad. Sci. U.S.A. 96*, 6953−6958.
6. Lee, K. J., Mao. S., Sun, C., Gao, C., Blixt, O., Arrues, S., Hom. L. G., Kaufmann, G. F., Hoffman, T. Z., Coyle, A. R., Paulson, J., Felding-Habermann, B., and Janda, K. D. (2002) Phage-display selection of a human single-chain fv antibody highly specific for melanoma and breast cancer cells using a chemoenzymatically synthesized G(M3)-carbohydrate antigen, *J. Am. Chem. Soc. 124*, 12439−12446.
7. Ravn, P., Danielczyk, A., Jensen, K. B., Kristensen, P., Christensen, P. A., Larsen, M., Karsten, U., and Goletz, S. (2004) Multivalent scFv display of phagemid repertoires for the selection of carbohydrate-specific antibodies and its application to the Thomsen-Friedenreich antigen, *J. Mol. Biol. 343*, 985−996.
8. Neumaier, M., Shively, L., Chen, F. S., Gaida, F. J., Ilgen, C., Paxton, R. J., Shively, J. E., and Riggs, A. D. (1990) Cloning of the genes for T84.66, an antibody that has a high specificity and affinity for carcinoembryonic antigen, and expression of chimeric human/mouse T84.66 genes in myeloma and Chinese hamster ovary cells, *Cancer Res. 50*, 2128−2134.
9. Wu, A. M., Chen, W., Raubitschek, A., Williams, L. E., Neumaier, M., Fischer, R., Hu, S. Z., Odom-Maryon, T., Wong, Y., and Shively, J. E. (1996) Tumor localization of anti-CEA single-chain Fvs: improved targeting by non-covalent dimers, *Immunotechnology 2*, 21−36.
10. DiGiacomo, R. A., Xie, L., Cullen, C., and Indelicato, S. R. (2004) Development and validation of a kinetic assay for analysis of anti-human interleukin-5 monoclonal antibody (SCH 55700) and human interleukin-5 interactions using surface plasmon resonance. *Anal. Biochem. 327*, 165−175.

11. Li, S.-L., Liang, S.-J., Guo, N., Wu, A. M., and Fujita-Yamaguchi, Y. (2000) Single-chain antibodies against human insulin-like growth factor I receptor: expression, purification, and effect on tumor growth, *Cancer Immunol. Immunother. 49*, 243−252.
12. Wu, A. M., Tan, G. J., Sherman, M. A., Clarke. P., Olafsen, T., Forman, S. J., and (2001) Raubitschek, A. A. Multimerization of a chimeric anti-CD20 single-chain Fv-Fc fusion protein is mediated through variable domain exchange, *Protein Eng. 14*, 1025−1033.
13. Ono, K., Kamihira, M., Kuga, Y., Matsumoto, H., Hotta, A., Itoh, T., Nishijima, K., Nakamura, N., Matsuda, H., and Iijima, S. (2003) Production of anti-prion scFv-Fc fusion proteins by recombinant animal cells, *J. Biosci. Bioeng. 95*, 231−238.

BI0618767