Original Article

Production of a human antibody fragment against the insulin-like growth factor I receptor as a fusion protein

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ABSTRACT: The aim of this study was to isolate single-chain variable fragments (scFvs) against human insulin-like growth factor I receptor (IGF-IR) from a phage library displaying human scFvs. Isolated scFvs-displaying phages showed affinity for IGF-IR in comparison to the control. Expression of scFv proteins in *Escherichia coli* for further characterization, however, proved extremely difficult. Alternatively, the scFv protein was expressed as a fusion protein with a maltose-binding protein (MBP) that is a highly soluble *E. coli* protein. The MBP-scFv fusion protein expressed in a soluble form in *E. coli* was purified to homogeneity by two-step affinity chromatography. The resulting MBP-scFv exhibited affinity for IGF-IR and structurally-related insulin receptor (IR). These results suggest both that MBP-scFv fusion proteins are practical alternatives to isolating scFv proteins for further characterization and that successful isolation of human scFvs against a specific protein of interest requires vigorous screening in the early stages. Such screening is accomplished by using two independent screening methods such as measuring binding to IGF-IR but not to IR by ELISA or measuring competitive binding by IGF-I in addition to binding to IGF-IR alone.

Keywords: Therapeutic antibody, Single-chain antibody, Insulin-like growth factor I receptor, Phage display, Maltose-binding protein fusion

1. Introduction

Insulin-like growth factor I receptor (IGF-IR) plays an essential role in cancer growth, progression, and metastasis (*1-3*). IGF-IR is overexpressed in a variety of malignant tumors and also plays a role in hormone-independent growth of breast and prostate cancers (*4,5*). IGF-IR is therefore considered to be a good target molecule for cancer therapy. Several anti-cancer strategies have been developed such as anti-sense RNA (*6*), tyrosine kinase inhibitors (*7*), and mAbs (*8*), but anti-IGF-IR mAbs are probably the best anti-tumor therapeutics for several reasons. First, the antibodies bound to the receptor result in inhibition of ligand-induced phosphorylation of β subunits followed by silencing of down-stream signal molecules. Secondly, antibodies induce receptor clustering due to their bivalency. The antibody-receptor complex is then internalized into endosomes and then to lysosomes, where the receptors are thought to degrade. This process, down-regulation of IGF-IR, was first demonstrated in breast cancer cells (*9,10*) and is responsible for causing the refractoriness of cancer cells to IGF-I stimulation and inducing apoptosis. Thirdly, IGF-IR antibodies can recruit effector functions, including ADCC through FCγR and complement fixation (*11*).

Several approaches to producing therapeutic antibodies are now available, that is, CDR grafting from the mouse variable region to a human frame (*12*), immunizing transgenic mice carrying human antibody gene loci (*13*), and screening of phage display libraries *in vitro* (*14*). Of these approaches, the phage display screening method is a powerful tool for producing scFv or Fab fragments *in vitro* in a short period of time. The major drawback associated with this method is, however, difficulty in readily producing soluble scFv proteins in *E. coli* transfected with original phages. For example, previous studies reported aggregations of scFvs in the periplasmic space of *E. coli* (*15,16*). Several methods of improving solubility have been evaluated such as use of different *E. coli* strains

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(*17*), changes in the vector construction (*18*), and introduction of a new tag (*19*).

The current authors previously reported that 1H7 scFv-Fc consisting of scFv derived from anti-IGF-IR mAb 1H7 and a human IgG1 Fc domain had an inhibitory effect on tumor growth *in vivo* (*9,10*). In an attempt to obtain more effective as well as humanized anti-IGF-IR Abs, a phage library displaying human scFvs was screened in this study using a human recombinant IGF-IR extracellular domain as an antigen. Since difficulties in expressing scFv proteins from phage-infected *E. coli* were encountered as anticipated, a scFv gene was fused to the gene for maltose-binding protein (MBP) in order to produce scFv proteins of interest in a soluble form (*20*). MBP-scFv expressed was purified by two-step affinity chromatography and was shown to bind to the antigen in a dose-dependent manner. Therefore, MBP fusion protein can be used to characterize scFv in *in vivo* experiments. The purified MBP-scFv protein was also found to bind to the insulin receptor, which indicated the presence of common epitopes for isolated scFvs.

2. Materials and Methods

2.1. *Materials*

E. coli strains used were the suppressor strain TG1, and the nonsuppressor strain TOP10F' from Invitrogen (CA, USA). *E. coli* JM109 was the suppressor strain from Takara Bio (Shiga, Japan). *E. coli* XL1-Blue was the suppressor strain from STRATAGENE (CA, USA). Recombinant human extracellular IGF-IR (rhIGF-IR) and IR (rhIR) were purchased from R&D Systems Inc. (MN, USA). Helper phage M13KO7, HRP/anti-M13 conjugate, HRP/anti-E tag conjugate, and His MicroSpin Purification Module were from GE Healthcare Bio-Sciences Corp. (NJ, USA). The plasmid vector pMAL-p2E that encodes MBP, anti-MBP mAb, and Amylose Resin High Flow were purchased from New England Biolabs (MA USA). All DNA primers used in this study were designed accordingly and ordered from Nihon Gene Research Laboratories, Inc. (Sendai, Japan). A control phage named 1H7 displaying mouse scFv specific for IGF-IR was constructed as previously described (*21*).

2.2. *Selection of IGFIR-binders from a phage library-displaying human scFvs by panning*

A phage library representing over 10^{11} independent clones that displayed human scFvs was constructed and used to screen anti-IGF-IR scFvs as previously described (*22*). Phage clone selection was basically carried out according to previously published procedures with some modifications (*22,23*). For the first panning, 24 wells of a 96-well plate were coated with rhIGF-IR 50 ng/50 µL in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS) and incubated at 4ºC overnight. The wells coated with rhIGF-IR were blocked by incubation with 150 µL of 3% bovine serum albumin (BSA) in TBS at room temperature (RT) for 2 h. After removal of blocking solution, the phage library was added to the wells and incubated at RT for 2 h. Unbound phages were washed away by incubation with TBS containing 0.2% Tween 20 (TBST) and TBS. For elution, 100 mM triethyl amine (TEA) solution was added to each well and the plate was incubated at RT for 10 min. TEA solution containing eluted phages was neutralized by adding 0.7 M Tris-HCl buffer, pH 7.4, containing 1.5% BSA solution. This elution step was repeated. *E. coli* TG1 cells were added to collected phage solutions and incubated at 37ºC for 1 h to allow phages to infect TG1 cells. Infected TG1 cells were spread out on LB (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1 mM NaOH) supplemented with 2% glucose and 50 µg/mL carbenicillin (LBGC) in plates and allowed to grow at 25ºC for 2 d until independent colonies formed. All resulting colonies were pooled and stored in SBS medium (3% tryptone, 2% yeast extract, 0.5% NaCl, and 20 mM Tris-HCl buffer, pH 7) supplemented with 16% glycerol at -80ºC. For the second round of panning, this phage-infected *E. coli* stock was used to enrich IGF-IR reactive scFvs. Five hundred µL of this stock were added to SBS supplemented with 50 µg/mL of carbenicillin (SBSC) and cultured at 37ºC for 2 h followed by infection with 8.8×10^{10} pfu of M13KO7 helper phage by culturing at 37ºC for 1 h. To select double-infected and scFv-producing *E. coli*, 100 µg/mL chloramphenicol and 50 µg/mL kanamycin were added to the *E. coli* solution followed by culturing at 25ºC for 2 d. The resulting phage preparation was precipitated in 4% polyethylene glycol/0.5 M NaCl (PEG precipitation) and resuspended in TBS containing 1.5% BSA and 0.2% Tween 20 followed by treatment with Benzonaze (Novagen) to digest any unnecessary DNA. The prepared phages were subjected to the second round of panning following the above procedure with some modifications. For the second to fourth round of panning, a longer elution time and fewer antigen-coated wells were used as the panning process advanced. Furthermore, *E. coli* cells infected with phages eluted after the second and third rounds of panning were directly added to SBSC medium and then subjected to the helper phage-rescuing procedure as described above.

2.3. *Screening for phages displaying human scFvs against IGF-IR*

After four rounds of panning, the concentrated phages were subjected to dilution and infection to logarithmically growing *E. coli* XL1-Blue strain.

Infected cells were plated on LBGC agar plates and incubated at 25ºC for 2 d. Each colony was picked up and cultured in 150 µL of SBSC supplemented with 2% glucose in 96-round well plates. After infection with helper phage, phage clones were obtained by adding 100 µg/mL chloramphenicol and 50 µg/mL kanamycin and culturing at 25ºC for 2 d, followed by centrifugation at 1500 g for 10 min. Fifty µL of each supernatant were collected and mixed with 100 µL of 3% BSA in TBS. These supernatants containing phages were used for ELISA.

2.4. *Screening phage clones for scFv inserts by PCR and DNA sequencing*

ScFv gene inserts were amplified from respective XL1-Blue colonies infected with individual phages by PCR with a primer set (Forward Cm-f: 5'-TGTGATGGCT TCCATGTCGGCAGAATGCT-3', Reverse g3-r: 5'-G CTAAACAACTTTCAACAGTCTATGCGGCAC-3') in 30 µL of reaction mixture. After preheating at 94ºC for 2 min, PCR was carried out with 35 cycles under conditions of denaturing at 94ºC for 20 sec, annealing at 53ºC for 20 sec, and extension at 68ºC for 1 min. After purification and confirmation on 1% agarose gel electrophoresis, the resulting scFv genes were subjected to DNA sequencing. DNA sequences of scFvs were determined using a 3730 DNA Analyzer (Applied Biosystem, Foster City, CA).

2.5. *Expression of human scFvs*

E. coli TOP10F'-FS, non-suppressor strain, harboring a chaperone/repressor vector carrying a spectinomycin resistance gene, as was produced by Takayanagi *et al*. (manuscript in preparation) was infected with phages, followed by selection on LBGC agar plates in the presence of 50 µg/mL of spectinomycin (LBGCS). Once colonies were confirmed to have the scFv gene by colony PCR, *E. coli* cells were cultured overnight in 5 mL of 2 × YT medium containing 50 µg/mL carbenicillin, 50 µg/mL spectinomycin (2 × YTCS), and 1% glucose at 25ºC. The solution cultured overnight was then cultured in 40 mL of freshly-prepared 2 × YTCS for 1 h at 30ºC, followed by induction with 1 mM IPTG at 30ºC for 5 h. Periplasm fractions collected as described above were analyzed by ELISA.

2.6. *Construction, expression, and purification of a MBP-scFv fusion protein (MBP-scFv)*

Phagemids were prepared from phage-infected *E. coli* with a Miniprep Kit (Sigma) and used as templates for genetic application. Construction, expression, purification, and analysis of MBP-scFv were basically carried out according to previously published procedures (*24*), except that a signal sequence was included in the current construct so that MBP-cFv would be expressed in the periplasmic space of *E. coli*. A candidate scFv gene, 5E4, was amplified by PCR with primers (MBP-scFv forward: 5'-TCCGG AATTCTCTAGAGCCATGGCCCA-3' and MBP-scFv reverse: 5'-CCAATGCATTGGTTCTGCAGT TATCAGTGGTGGTGGTGGTGGTG-3') under the following conditions: preheating at 94ºC for 2 min, 35 cycles of denaturing at 94ºC for 1 min, annealing at 54ºC for 1min, and extension at 68ºC for 1 min. The amplified scFv gene and pMAL-p2E vector encoding MBP were digested by restriction enzymes, *EcoR I* and *Pst I*, followed by ligation reaction with Ligation High (TOYOBO) at RT for 1 h. The ligated scFv gene/vector DNA was precipitated by ethanol and dissolved in ultra-pure water. *E. coli* JM109 strain was transformed with a resulting vector and cultured on LBGC plates. The resulting colonies were checked to confirm the presence of the inserted gene by colony PCR. The confirmed colonies were cultured in LBGC medium at 25ºC overnight. The cultured *E. coli* was diluted 10-fold with LBGC medium. After culturing at 25ºC for 2.5 h, 0.3 mM isopropyl-thio-β-D-galactopyranoside (IPTG) was added to the *E. coli* culture to induce expression of the MBP-scFv protein. Cells were collected after culturing at 20ºC for 5 h, from which the periplasm fraction was prepared by means of mild osmotic shock. The periplasm extraction collected was subjected to purification by two-step affinity chromatography. Briefly, the periplasm fraction was passed through amylose resin. The eluates were further purified with a His-spin column (Ni^{2+} column). Each purification step was analyzed for the amount and content of protein by the Bradford method and SDS-PAGE (4-20%), respectively. In parallel, MBP protein was purified by amylose resin from *E. coli* transformed with pMAL-p2E vector itself. The binding specificities of the purified MBP-scFv and MBP proteins were analyzed by ELISA.

2.7. *Evaluation of specificity by ELISA*

Binding of phage antibodies or soluble MBP-scFv proteins to IGF-IR was evaluated by ELISA. Each well of a 96-well plate was coated with 500 ng/50 µL of rhIGF-IR or rhIR followed by incubation for 2 h at RT. Antigen-coated wells and control wells were blocked by incubation overnight with 150 µL of 3% BSA/TBS at 4ºC. The wells were then incubated with 50 µL of phage antibodies or soluble scFv proteins at RT for 2 h. The wells were washed 3 times with 150 µL of TBST. For detection of phage antibodies, HRP/anti-M13 conjugate (1:2,500 dilution) was used as a second antibody. For detection of MBP-scFv, anti-MBP mAb (1:1,000 dilution) and HRP/anti-mouse antibody conjugate (1:1,000 dilution) were used as a second and

a third antibody, respectively. For detection of scFv proteins, HRP/anti-E-tag conjugate (1:2,000 dilution) was used as a second antibody. The wells were washed 7 times with TBST and then 3 times with TBS. Peroxidase activity was detected by reaction with 100 µL of ABTS/H_2O_2 for 30 min and termination with 1% oxalic acid. The absorbance at 415 nm was measured by a BIO-RAD plate-reader.

2.8. *Characterization of phage antibodies by surface plasmon resonance (SPR)*

SPR analysis was carried out at 25ºC using 10 mM HEPES, pH 7.4, containing 150 mM NaCl, and 0.005% surfactant P20 (HBS-P buffer) as a running buffer. Binding properties of phage antibodies were determined using a Biacore X (Pharmacia Biosensor AB, Uppsala, Sweden). A CM3 sensor chip was equilibrated overnight with the running buffer before use. Immobilization of the antigen on the sensor chip was achieved by injecting 50 µL of 10 mM sodium acetate buffer, pH 3.8, containing 500 µg/mL of rhIGF-IR *via* amine groups using the Amine Coupling Kit (Pharmacia Biosensors) as previously described (*25*). Binding of phage antibodies displaying 2A1, 3E2, 3H5, and 4C5 scFv was analyzed at two or three different concentrations as indicated in the figure legend. M13KO7 helper phage, which does not display scFv, served as a negative control.

3. Results

3.1. *Panning and screening of anti-IGF-IR human scFv-displaying phages*

A phage display library consisting of more than 10^{11} independent clones was subjected to four rounds of panning against rhIGF-IR as an antigen (Figure 1). Of 419 independent clones screened by ELISA, twenty-three positive clones were found to show ELISA positivity with S/N of > 2. Typical results of ELISA are shown in Figure 2A. ScFv genes, amplified from 23 clones by PCR as indicated by the examples in Figure 2B, were subjected to DNA sequencing analyses, which revealed that 4 clones were identical. Thus, 20 independent clones were obtained as candidate phages presenting anti-IGF-IR scFvs. Amino acid sequence alignments of VH and VL of all 20 scFvs are shown in Tables 1A and B, respectively. These scFvs were clearly derived from different clones with diverse origins.

3.2. *Initial characterization of phage antibodies by SPR*

SPR analyses were carried out to determine whether or not phages displaying "anti-IGF-IR" scFvs have binding affinities for IGF-IR. Figures 3A, B, C, and D

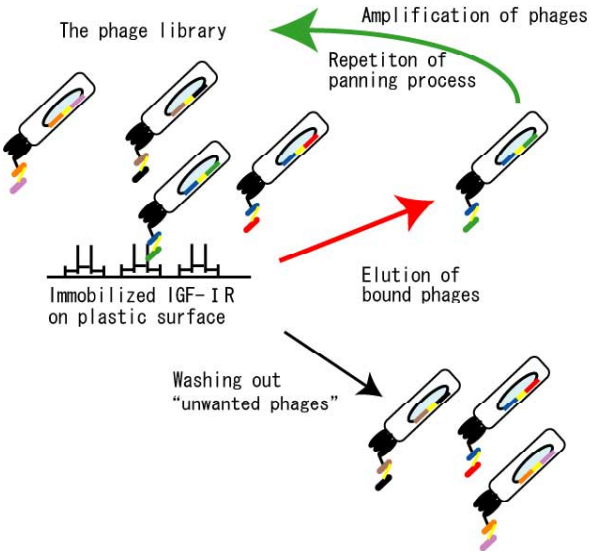

Figure 1. Schematic panning procedure for isolation of human scFvs displayed on phages. Phage antibodies were added onto wells coated with rhIGF-IR. After washing, bound phages were eluted and amplified in *E. coli*. This process was repeated four times to concentrate antigen-specific phage clones.

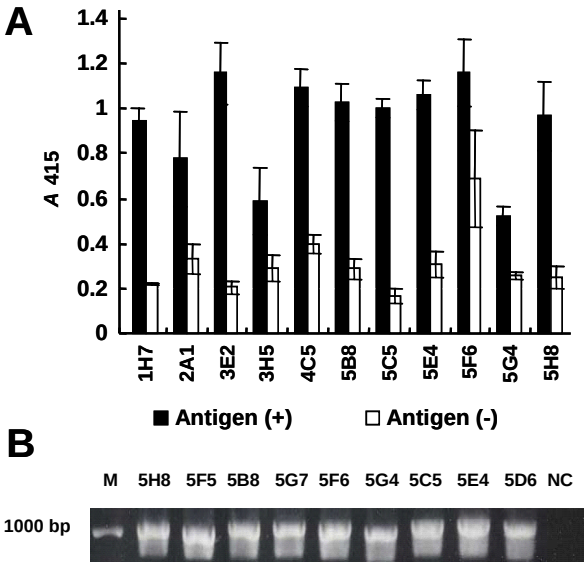

Figure 2. Analyses of candidate phage antibodies screened by human IGF-IR from a phage library displaying scFvs. (A) Binding of isolated phage antibodies to IGF-IR as measured by ELISA. Each phage clone (1×10^{12} cfu/mL) was assayed for its affinity against immobilized rhIGF-IR (Antigen +) or BSA only (Antigen –) as described in the Methods. 1H7 is a control phage displaying mouse scFv prepared from anti-IGF-IR mAb 1H7-producing hybridoma as described (*21*). The error bars represent the standard deviation calculated from replicates ($n = 3$). (B) PCR amplification of scFv genes in the isolated clones. ScFv genes were amplified by colony PCR using specific primers as described in the Methods. PCR products were analyzed by 1% agarose gel electrophoresis.

show sensorgrams at different concentrations of phages displaying 2A1, 3E2, 3H5, and 4C5 scFv, respectively. Although these data are merely qualitative, the resulting sensorgrams clearly indicated that the phage antibodies bound to immobilized IGF-IR in a dose-dependent manner. In addition, M13KO7 helper phage, which served as a negative control, displayed no signal (data not shown), indicating that all phage antibody affinity

Table 1A. Multiple alignment for deduced amino acid sequences of 20 anti-IGF1R scFv VH fragments

2A1	QVQLVESGPDVKKPGASVKVSCKTSGYTFTD--HYIHWVRQAPGQGLEWMGWVTPQSASTNYADKFQSRVTMTRDTSINTAYMELSGLTSDDTAIYYCAR-GERT-------PLERW-LDPWGQGTLVTVSS--------
2A7	QVQLVQSGGGLEQPGGSLRLSCAASGLTFSS--YAMSWARQAPGKGLEWVSSISSSGGTTYYADPVKGRFTISRDNSKNTLYLQMNSLRAEDTALYYCASQKSVCTDGICYKDYY YYGMDVWGQGTTVTVSS--------
2D9	QVQLLETGGGVVQPGRSLRLSCEASGSSFSH--YAIQWVRQAPGKGLEWVAVISFDGRERYYADSVKGRFAVSRDNSKNTLHLQMNSLRPEDTAVYYCAREMYPSTTVI-----SPD GMDVWGLGTTVTVSSASTKGPSS
2G5	QVQLVESGGGVVQPGESLRLSCAASGFIFSR--YGMQWVRQAPGKGLEWVAFIPYDGSNKYYVDSVKGRFTVSRDNSKNTLYLQMNSLRGEDTAVYHCAICRDG----------YNPL DHWGQGTLVAVSSASTKGPSS
3A5	QVQLVESGGGLVQPGGSLRLSCVASGFSFSP--YSMNWVRQAPGKGLEWLSYISGSSGTIYYAESVKGRFTISRDNGKNSLYLQMNRLTVDDTALYYCARESGTG-----PTHYYSH GMAVWGQGTTVTVSS--------
3A6	QVQLVESGGDLIQPGGSLRLSCAASGFIVSS--KYMTWVRQAPGKGLEWVSVIDS-GGTTYYANSVKGRFTISRDHSKNTLYLQMNSLRAEDTAVYYCVRDSSS----------SGLDY WGQGTRVTVSSGSASAPT-
3C5	QVQLVESGGALVKPGGSLRLSCTASGFSLSD--YNINWIRQAPGKGLEWVSSFSSGGKTKMYANSVRGRFTVSRDSAKNSLFLQMNSLTADDTAMYYCASPYIRG--------IVAYYF HYWGHGTLVTVSS--------
3C10	QVQLLETGAEVKKSGASVKVSCKASGFGLSD--YFMHWVRQAPGQGLEWMGWINPNNGGAKYAQKFQGRVTMTRDTSINTVYMELTRLRPDDTAIYYCAREGQEG-------YGG DW-FDPWGQGALVTVSS--------
3E2	QVQLQQSGPGLVRPSETLSLTCTVSEGSFRS--YYLSWIRQSAGKGLEWIGRMYL-NGKTNYNPSLRSRVIMSVDTSKKQFSLNLSSVTAADTAVYYCATDRGW-------ATSSQG-M DVWGQGTTVTVSS--------
3G5	QVQLQESGGGLVQPGGSLRLSCAASGFKFSD--YWMHWVRQAPGKGLMWVSRINSDGSSTTFAESVKGRFSMSRDNAKNTLYLQMNSLRGDDAAVYYCVR--DS----------FTA LDLWGQGTLVTVSSASTKGPSS
3G8	QVQLVESGAEVKRPGSSLRLSCKAFGGSFSS--YAFSWLRQAPGQGPEWMGRIIPIVGLPTYTSNFQGRISISADTSTRTVFMDLNSLNSDDAAVYFCARESSRN-------SGVGYYFD LWGQGSLVTVSS--------
3H5	QVQLVESGGGVVQPGRSLRLSCAASGFTFSR--HEMHWVRQAPGKGLEWVALISNDGGSNYYADFVKGRFTISRDNSKTSLHLQMNSLRPDDTAIYYCARDTVG----------VGM DVWGQGTTVTVSS--------
4C5	QVQLQQSGPGLLKPSQTLSLTCTVSDGSISSGSHYWSWIRQAPGKGLEWIGHIFY-SGVTYYTPSLKSRLTMSADTSKNQFSLRLTSVTAADTAVYYCARQICF-------GASCS--FDS WGQGALVTVSS--------
5B8	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSS--YAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARANYDF-------WSGYTSG GPWGQGTLVTVSS--------
5C5	QVQLVESGGDLVQPGGSLRLSCAASGFTFRD--YAMSWVRQAPGKGLEWVSTSSGSGSNIYYADSVKGRFTISRDNSKNMVYLQMHSLRADDTATYYCVKGG----------YYYH MDVWGQGTTVTVSS--------
5D6	QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVSWIRQPPGKALEWLAHIFS-NDEKSYSTSLKSRLTISKDTSKSQVVLTMTNMDPVDTATYYCGV----------ASRE---YDYW GQGTLVTVSS--------
5E4	QVQLVETGAEVKKPGASVRVSCKPSGYNFSD--YFLHWVRQAPGQGLEWMGWINPRSGDTTYAQTFLGRVTLTSDTSINTAYMELSSLTSDDAAVYYCARDIITG-------ALYYYA MDVWGQGTTVTVSS--------
5F6	QVQLVESGAEVKKPGSSVKVSCTASGGTFRS--YVFSWVRQAPGQGLEWMGGIIPVFGTPKYAQKYQGRVSITADESTSTAYMELSSLRSEDTAVFYCAVGALLP-------PTYYYGM DVWGQGTTVTVSS--------
5G4	QVQLVESGGGLVKPGGSLRLSCAASGFTFSD--YYMSWIRQAPGKGLEWVSFISAGGSIISYADSVKGRFTVSRDDAKNSLYLQMNSLRAEDTAVYYCFG----------DYGVDV WGRGTTVTVSS--------
5H8	QVQLQQSGPGLVKPSETLSLTCIVSGDSMSSDYYYWSWLRQPPGKGLEWIGNVFH-SGFPYYNPSLHNRATISIDTSESRFSLKLTSVTAADNAVYYCARSLHE-------YGDYVGW LDPWGQGTLVTVSS--------

Table 1B. Multiple alignment for deduced amino acid sequences of anti-IGF1R scFv VL fragments

2A1	QSVLTQPP-SVSGTPGQRVTISCSGSSSNIGSST----VNWYHQLPGAAPKLLIYNNDQRPSGVPDRFSGSK--SGTSASLAISGLQSEDEADYYCAAWDGSLSGR--VFGGGTKLTVL
2A7	NFMLTQPH-SVSESPGKTVTMSCTGSGGSIATSY----VQWYQQRPGSVPTTVIYEDDQRPSGVPDRFSGSVDSSSNSASLTISGLKSEDEADYYCQSYDG-SNVI---FGGGTKVTVL
2D9	QTVVTQEP-SLTVSPGGTVTLTCASSTAAVTTAYY---PNWFQQKTGQAPTPLIYSTDNKHSWTPARFSGSL--LGGKAALTLSRVQPDDEADYYCLLWFG-GAWV---FGGGTKLTVL
2G5	QSVLTQPP-SASGTPGQRVTISCSGSSSNIGSNY----VYWYQQLPGTPPKLFIYRNNERPSGVPDRFSGSK--SGTSASLAISGLRSEDEADYYCAAWDDSLSGR--LFGGGTKLTVL
3A5	QPGLTQPP-SVSLSPGQTASITCSGDK--LEEKY----VSWYQQKPGQSPVLVIYQDNRRPSGTPERFSGSN--SGNTATLTITGTQAIDEADYYCQAWDT--FTVG--FGGGTKLTVL
3A6	NFMLTQPH-SVSESPGKTITISCTRSSGNIVSNY----VQWYQQRPGSAPTTVIYEDDRRPSGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYCQSSHT-SYVV---FGGGTKLTVL
3C5	QSVVTQPP-SVSGAPGQRVTISCTGSSSNIGTGYD---VQWYQQLPGTAPKLLIYDNNNRPSGVPDQFSGSK--SGTSASLAITGLQAEDEADYYCQSYDSSLSGHNYVFGTGTKLTVL
3C10	NFMLTQPH-SVSESPGKTVTISCSGISGDIG-DY----VQWYQQRPGSAPTTVIYENDQRASGVPDRFSGSIDRTSKSASLTISGLKTDDEADYYCQSYAGDTLWV---FGGGTKLTVL
3E2	EIVLTQSPATLSLSQGERATLSCRASQNFGS-----YYLAWYQQKPGQAPRLLIYGASTRATGIPDRFSGSG--SETDFTLTISGLDPEDSATYYCHQYAG---PPGGTFGQGTKVEIK
3G5	EIVLTQSPDSRAVSLGEGATIDCKSSQSLLKSSNNKNYLGWYQQKPGQPPKLLIYWASTRESGVPDRFSGSG--SGTDFTLTISSLQAADVAVYYCHQYYN---TP-YTFGQGTKLEIK
3G8	EIVLTQSPDTLSLSPGERATLSCRASQSVTD-----TYLAWYQQRPGQAPRLLIYGASSRATGIPDRFSGSG--SGTDFTLTISRLEPEDFAVYYCQQHG----TSPFTFGPGTKVDIK
3H5	DIVMTQSPSSLSASVEDRVTITCRASQGIRN------DLGWYQQKPGKAPQRLIYAASSLQSGVPSRFSGSG--SGTEFTLAISSLQPEDFATYYCQQSYS---TP-ITFGQGTRLEIK
4C5	DIVLTQSPATLSLSPGERATLFCRASQSVG------SYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGNG--SGTDFTLTISSLEPEDFALYYCQQP-----TMGVSFGPGTKVDIK
5B8	DIVMTQTPDSLAVSLGETATITCKSSRSVYFRSNSGDFLAWYQQKQGQPPKLLISWASTRESGVPDRFSGSG--SGTDFTLTISRLQSEDAAVYFCQQYYD---TP-PTFGPGTKEDIK
5C5	DIRVTQSPDSLAVPLGERATINCKSSQSILYSPNNKNYLGWFQQKPGQPPKLLIYWASTRESGVPDRFSGSG--SGTDFTLTISSLQAEDVAVYYCQQYYG---SPPITYGQGTRLEIK
5D6	DIRVTQSPSSLSASVGDRVTITCRASQSIHR------YLNWYQEKPGKAPKLLIYAASSLQSGVPSRFSGSG--SGTEFTLAISSLQPEDFATYYCQQSYS---TP-ITFGQGTRLEIK
5E4	QPVLTQPP-SASGTPGQRVTISCSGSSSNIGSNT----VNWYQQLPGTAPKLLIYSNIQRPSGVPDRFSGSK--SGTSAALAISGLQSEDE AEYYCAAWDDSLNGL--LFGGGTKLTVL
5F6	NFMLTQPP-SASGTPGQRVTVSCSGSSSNIGTNF----VYWYQQVPGAAPRLIIYRNDQRPSGVPDRFSGSK--SGTSASLAISGLRSEDKADYYCSAWDDRLDGP--MFGGGTKLTVL
5G4	DIRVTQSPSSLSASVGDRVTITCRASQSIIT------YLNWYQQKPGKAPKLLIYATSSLQSGVPSRFSGSG--SGTDFTLTISSLQPEDFATYYCQHLHS---YP-ITFGQGTRLEIK
5H8	SSELSQDP-AVSVALGQTVKIICQGDS--LRTFY----AGWYQQKPGQAPTLVVYDKNNRPSGIPDRFSGSK--SGNTAFLTITGAQAEDEADYYCISRDISGNHWV—FGGGTKLTVL

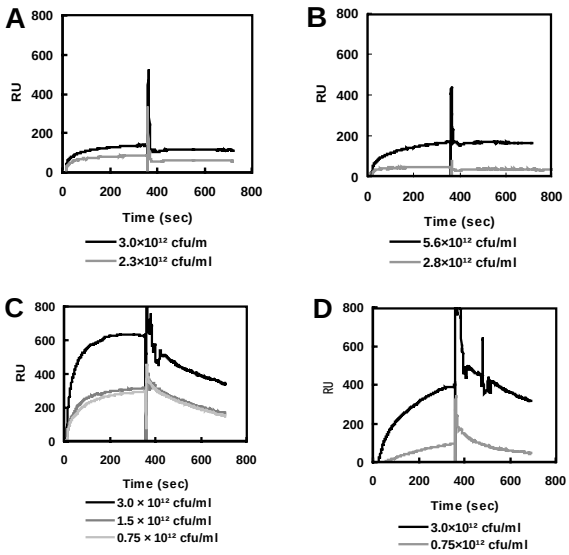

Figure 3. SPR analyses of scFv-presenting phage antibodies. Phage antibodies at indicated concentrations were passed over the IGF-IR-immobilized CM3 chip at a flow rate of 5 μL/min as described in the Methods. The sensorgrams are 2A1 (A), 3E2 (B), 3H5 (C), and 4C5 (D) phage antibodies, respectively.

is attributable to the scFv portions and not due to the phage itself.

3.3. *Expression of a soluble scFv protein from a phage-displaying human scFv*

Although different *E. coli* strains transfected with phages derived from the original phage library were subjected to induction of scFv protein expression by IPTG, levels of expression of scFv proteins in this modified system were much lower than in the conventional system (*21*). SDS-PAGE/CBB staining (Figure 4A) and Western blotting/anti-E tag Ab immunostaining (Figure 4B) revealed an expression profile typical of scFv proteins produced from TOP10F'-FS infected with phages. The immunostaining did not reveal any apparent bands with ~30 kDa scFvs, indicating that respective scFv proteins were not produced.

3.4. *Expression, purification and evaluation of MBP-scFv*

To thus produce and purify scFv proteins, one of the isolated scFvs was expressed as a MBP-fused protein in *E. coli* and was expected to be a highly soluble and stable protein (*20*). The 5E4 scFv gene, selected as a model scFv gene encoding a typical scFv sequence, was inserted into pMAL-p2E vector as illustrated in Figure 5A. *E. coli* JM109, transformed with pMAL-p2E-5E4, was cultured with IPTG to induce the 5E4 MBP-scFv. Periplasm fractions were collected and subjected to two step-affinity chromatography purification. Each purification step was monitored

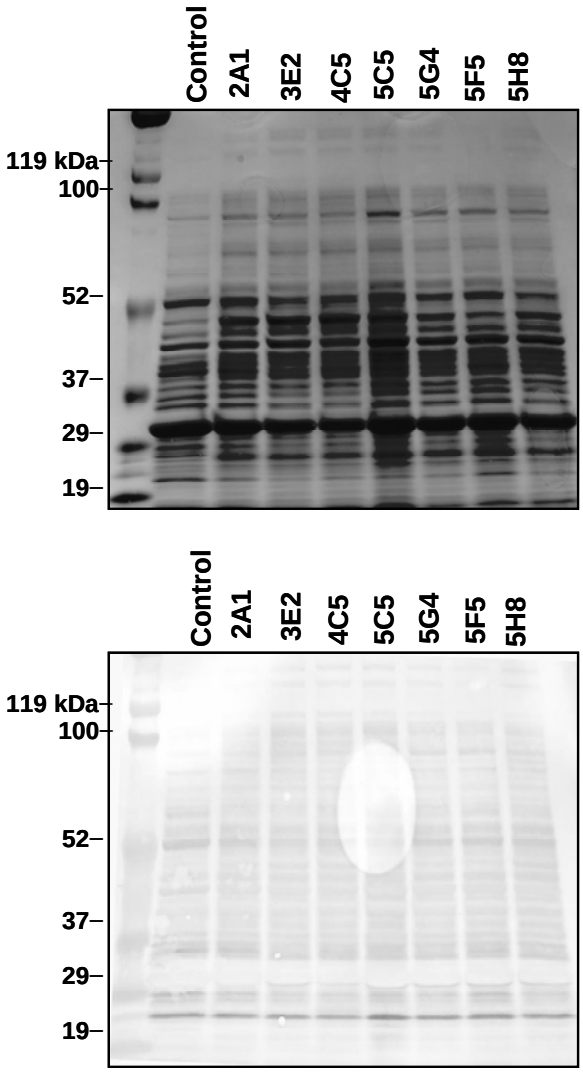

Figure 4. SDS-PAGE/Western blotting analysis of expression levels of isolated scFv proteins. Periplasm fractions (10 μg/lane) extracted from *E. coli* infected with phages were subjected to SDS-PAGE/CBB stain (Figure 4A) or blotted onto a PVDF membrane that was immunostained with HRP-anti-E tag Ab (Figure 4B).

by SDS-PAGE (Figure 5B). Both MBP and MBP-scFv proteins were recovered after the first affinity chromatography step (Figure 5B, lane 3), but only the MBP-scFv fusion protein was purified to apparent homogeneity by the second affinity chromatography step (Figure 5B, lane 5). The yield of purified MBP-scFv was 0.3 mg from 1L culture. As a control, MBP protein was expressed, purified, and analyzed by SDS-PAGE (Figure 5A, lane 6). The binding affinity of MBP-scFv for IGF-IR was evaluated by ELISA. As seen in Figure 6A, the purified MBP-scFv bound to IGF-IR in a dose-dependent manner whereas the purified MBP protein did not bind to IGF-IR, indicating that the binding of MBP-scFv to IGF-IR was *via* its scFv domain. Further analysis of MBP-scFv revealed cross-reactivity to rhIR (Figure 6B), which shares sequence and structural similarities. This result implied that the scFv recognizes the epitopes that are shared by IGF-IR and IR.

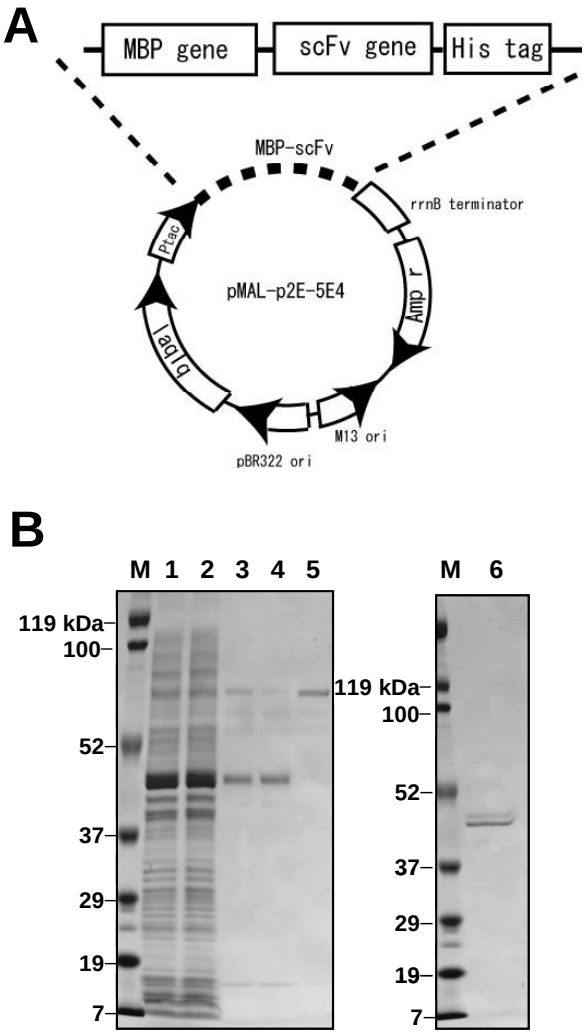

Figure 5. Construction, expression, and purification of anti-IGF-IR MBP-scFv. (A) Schematic representation of the pMAL-scFv vector into which the scFv gene (5E4) with a His tag sequence was inserted. (B) SDS-PAGE analysis of purification steps. The MBP-scFv protein expressed in the periplasm of *E. coli* was first applied to an amylose resin column. The eluates were further purified with a Ni^{2+}-column. SDS-PAGE gel stained with Coomassie Brilliant Blue shows: lane 1, periplasmic fraction; lane 2, flow-through fraction of the amylose resin column; lane 3, the eluted fraction from the amylose resin column, lane 4, flow-through fraction of Ni^{2+}-column; lane 5, the eluted fraction from Ni^{2+}-column (purified MBP-scFv), and lane 6, purified MBP. Five µg of protein were loaded in lanes 1 and 2 whereas 2 µg of protein were loaded in lanes 3-6.

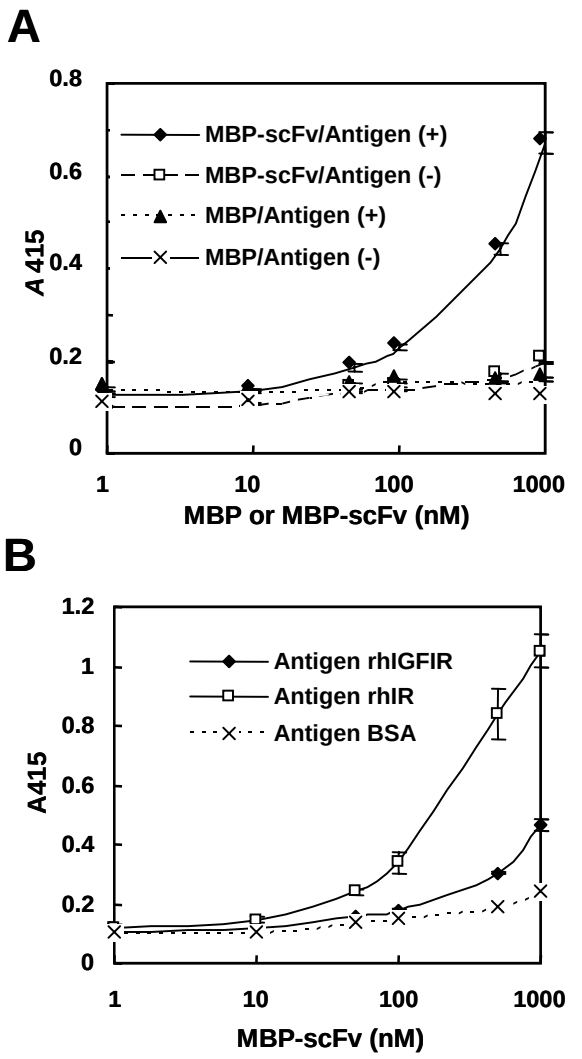

Figure 6. Binding of purified MBP-scFv and MBP to IGF-IR or IR. (A) Binding of the purified MBP-scFv and MBP to immobilized rhIGF-IR (antigen +) or BSA (antigen –) measured by ELISA. Binding of MBP-scFv and MBP at indicated concentrations was detected by an anti-MBP mAb followed by an anti-mouse IgG/HRP. (B) Binding of the purified MBP-scFv at indicated concentrations to immobilized rhIGF-IR (antigen IGF-IR), rhIR (antigen IR), or BSA (antigen BSA) was measured by ELISA as described above. The error bars represent the standard deviation calculated from replicates ($n = 3$).

4. Discussion

This study achieved successful isolation of phage antibodies bearing human scFvs against IGF-IR. As anticipated, expression of scFv proteins in the periplasmic space of phage-infected *E. coli* was so difficult that one of scFv proteins was fused with MBP. The resulting fused protein was successfully produced and purified by two-step affinity chromatography. MBP-scFv clearly exhibited binding affinity for IGF-IR whereas MBP did not bind to the antigen, which suggested that the IGFI-IR binding affinity of MBP-scFv protein was attributable to the scFv domain. Further experiments, however, revealed that MBP-scFv cross-reacted to rhIR, implying that the scFv recognizes the epitopes that are shared by IGF-IR and IR. This result highlights the importance of vigorous screening with the use of two or more independent approaches during isolation of candidate clones from a phage display library.

Since phage display technology provides genes that encode scFvs with specificity of interest, recombinant antibodies including completely human IgGs for therapeutic applications can readily be produced. However, the best clones must be chosen after completing initial characterization of scFv proteins from isolated phage clones by examining whether scFvs have high specificity and affinity for the antigen of interest. Usually, this can be achieved by inducing the production of scFv protein in phage-infected *E. coli*. Although scFvs that can be expressed in the

cytoplasm of cells have considerable biotechnological and therapeutic potential, the reducing environment of the cytoplasm inhibits the formation of disulfide bonds that are essential for correct folding and functionality of the antibody fragments. Thus, scFvs expressed in the cytoplasm are mostly insoluble and inactive. Alternatively, scFv proteins are often expressed in the periplasmic space, though this process is not always successful, as indicated by the current results (Figure 4). As a general approach to stabilizing scFvs for efficient functional expression in the cell cytoplasm, scFvs were expressed as C-terminal fusions with the *E. coli* MBP (*24*). A previous study demonstrated that MBP-fused scFvs are expressed at high levels in the cytoplasm of *E. coli* as soluble and active proteins regardless of the redox state of the bacterial cytoplasm, suggesting that MBP seems to function as a molecular chaperone that promotes the solubility and stability of scFvs. In this study, MBP-scFv protein was expressed in the periplasm, which should further facilitate stability of the expressed protein. In conclusion, this study demonstrated that MBP-scFv can be expressed as a recombinant human scFv in the periplasm of *E. coli*, which can be easily purified for further characterization.

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