Article

The Expression and Characterization of a Bifunctional Protein in *E. coli* for Autologous Erythrocyte Agglutination Test

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H antigen, the precursor of A and B antigens, belongs to Hh blood system in which it is the only antigen. H antigen distributes on all the human RBC surface except for Bombay phenotype and the copy number of H antigen on the surface of an adult RBC is approximately 1.7×10^6. These characteristics made H antigen the potential target molecule for the immunoassay and immunotherapy. A monoclonal antibody 2E8 against H antigen on the surface of erythrocyte had been prepared in previous work. Based on this antibody, the variable region genes of heavy and light chains (V_H and V_L) from 2E8 had been cloned by 5' RACE. The two variable region genes were spliced by overlap extension and assembled ScFv (V_H-linker-V_L) gene encoding the anti-H antigen named $ScFv_{2E8}$. According to the prediction of the three-dimension structure of $ScFv_{2E8}$ and C_H1 fragment from 2E8 and HIV-1 gp41 antigen peptide, we further constructed the $ScFv_{2E8}$-C_H1-gp41 fusion molecule. The recombinant $ScFv_{2E8}$-C_H1-gp41 gene was cloned into pET-his vector and expressed in BL21(DE3)plysS cells. The fusion protein was purified from the inclusion bodies. In a series of subsequent analyses, this fusion protein showed identical antigen binding site and activity with the parent antibody. Meanwhile, in mimic test, as the main ingredient of reagent for autologous erythrocyte agglutination test, the bifunctional protein could agglutinate the RBCs in the presence of HIV-1 gp41 antibodies using sera from HIV-infected individuals. *Cellular & Molecular Immunology*. 2008;5(4):299-306.

Key Words: agglutination, bifunctional protein

Introduction

Autologous erythrocyte agglutination test was a kind of whole blood cell agglutination for the assay of antibodies or antigens in the blood. The major characteristic of autologous erythrocyte agglutination test was simple and rapid in the process of blood assay. The results could be obtained within 2-5 min, while the sensitivity and specificity were comparable to enzyme-linked immunosorbent assay (ELISA). So, autologous erythrocyte agglutination test was suitable for the blood assay in emergency patients, high risk individuals and remote area populations.

Initially, the main ingredient of reagent for autologous erythrocyte agglutination test was prepared by chemical conjugation of an anti-human red blood cell (RBC) mAb with a peptide derived from HIV-1 envelope gp41 (1). But, for chemical methods, it had some problems in the process of preparation and utilization, such as the activity of mAb affected by the chemical reagents and long time of screening the proper molecules. To overcome the deficiencies, chemical conjugation techniques were improved by use of different enzymes, but they still maintained laborious and the yield of the conjugation was lower than expected (2). Though autologous erythrocyte agglutination test met some problems, it was still a creative design with good perspective, and could be developed for detection of a variety of antigens and antibodies (3).

Recombinant single chain Fv (ScFv)/Fab (antigen binding fragment) technology brought about the new hope of gaining the fusion protein as the main ingredient of reagent for autologous erythrocyte agglutination test. The advantage of this method was its easiness to get the fusion protein once the expression vector was constructed successfully. An example was then implemented for the preparation of monovalent ScFv fragment of anti-RBC antibody fused to immunodominant peptides derived from envelope glycoproteins gp41 of HIV-1 (4, 5). Later, for more functions, this group produced a Fab fusion protein carrying immunodominant epitopes of HIV-1 and HIV-2 fused to the C-terminal of Fd and LC in Fab (6). Recently, a more interesting attempt was to produce a cocktail formulation of

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Table 1. List of primers for the construction of the $ScFv_{2E8}$-C_H1-gp41 gene

Primer	Sequences
$2E8H_{BACK}$	5'-GGATCCCAGGTGCAGTTGAAGGAGTCAGGA-3'
$2E8L_{forCH1}$	5'-GGTGGAGTAGACGGCTTCGGTATTTCCAACTTTGTCCCCG-3'
C_H1_{back}	5'-CCGAAGCCGTCTACTCCACCCGGGACTAGTGCAAAGACCACTCCTCCG-3'
C_H1_{forgp1}	5'-TAACGTTCGACGGCCAGGATACGGCTAGCACAACCACAATCCCTGGGCAC-3'
gp2	5'-CAGATGCCCAGCAGCTGCTGATCTTTCAGATAACGTTCGACGCCAGGAT-3'
gp3	5'-GTGGTGCAGATCAGTTTGCCAGAGCAGCCCCAGATGCCCAGCAGCTGCTG-3'
gp4	5'-AGACGCATTCCACGGGACCGCCGTGGTGCAGATCAGTTTGCC-3'
gp5	5'-GCTAGCTTATCATCCTTCATGGTGGTGATGGTGAGACGCATTCCACGGGACCG-3'

The primers $2E8H_{BACK}$ and $2E8L_{forCH1}$ are for the cloning of $ScFv_{2E8}$ sequence, primers C_H1_{back} and C_H1_{forgp1} are for the cloning of C_H1 fragment, and primers gp2, gp3, gp4 and gp5 are for the cloning of HIV-1 gp41 peptide sequence. In the primers $2E8H_{BACK}$ and gp5, underlined sequences are the *Bgl* II and *Xba* I sites respectively.

reagent ingredient composed of several fusion proteins with different dominant epitopes of HIV (7). Those fusion proteins consisting of the monovalent Fab of anti-human RBC monoclonal antibody with immunodominant peptides derived from envelope glycoproteins gp41 of HIV-1, HIV-1 capsid protein p24 and gp36 of HIV-2, respectively (8, 9). In late studies, this group made a single molecule capable of simultaneous detection of both HIV-1 and HIV-2 in a drop of blood (10). Not only envelope glycoprotein gp41 of HIV-1, but also gp160 of HIV-1 was employed in this field (11).

Generally, the fusion protein derived from recombinant vector was expressed in *E. coli* in soluble form. But, the yield of the fusion protein was low because a small quantity of active proteins was released into culture supernatant. For another way to gain target protein, in this study, a recombinant bifunctional molecule ($ScFv_{2E8}$-C_H1-gp41) as the main ingredient of reagent for autologous erythrocyte agglutination test was designed and expressed in *E. coli*. The fusion protein was gained from the inclusion bodies and kept activity by denaturation and renaturation *in vitro*. Meanwhile, in mimic test, as the main ingredient of reagent for autologous erythrocyte agglutination test, the bifunctional protein could agglutinate the RBCs in the presence of HIV-1 gp41 antibodies using sera from HIV-infected individuals.

Materials and Methods

Cell line

A hybridoma strain 2E8 secreting monoclonal antibody (mAb_{2E8}) (12) against H antigen on the surface of human erythrocyte was cultivated at 37°C in 5% CO_2 atmosphere in RPMI 1640 medium with 10% fetal bovine serum, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.

Cloning of mAb_{2E8} variable region genes by 5' RACE

Total RNA was isolated from 2E8 hybridoma strain using TRIzol reagent (GIBCO) according to the manufacturer's protocol. cDNA was synthesized by extension of Oligo $(dT)_{15}$ primer using Superscript™-II Amplification System (Invitrogen) according to the protocol of manufacturer. The first strand was then poly-G tailed using terminal transferase (Promega). The tailed cDNA was amplified by PCR. The amplified system included 5 μl of 10× PCR buffer, 1 μl of 10 mmol/L dNTP mix, 2 μl of 50 pmol/L sense and antisense primers respectively, 2 U Superscript™-II polymerase, 1 μg cDNA and H_2O to total 50 μl. PCR was performed typically with 28 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C on a thermal cycler (Biometra). For amplification of variable domain *via* PCR with easy-A polymerase (Stratagene), the primers P_{ployC} 5'-CCC CCC CCC CCC CCC-3' and $V_{RACEHFOR}$ 5'-ACT AGT ACA ATC CCC TGG GCA CAA T-3' flanking the heavy chain, and P_{ployC} and $V_{RACEκFOR}$ 5'-CGT CAT GTC GAC GGA TCC AAG CTT CAA GAA GCA CAC GAC TGA GGC AC-3' were used, flanking the light chain. Amplified DNA was linked to pMD18-T vector (TaKaRa) and transformed into *E. coli* DH5α cells. Two independent clones of each variable domain were sequenced and compared for identity.

Tertiary structure prediction of $ScFv_{2E8}$-C_H1-gp41

Based on the sequence of $ScFv_{2E8}$, a C_H1 fragment from the hybridoma 2E8 (data unpublished) was cloned, and HIV-1 gp41 peptide sequence was searched from the published data. Three gene fragments ($ScFv_{2E8}$/C_H1/HIV-1gp41) theoretically constructed the new recombinant gene $ScFv_{2E8}$-C_H1-gp41. The sequence of $ScFv_{2E8}$-C_H1-gp41 gene was submitted to SWISS-MODEL by ExPASy; templates were obtained by searching in the ExNRL-3D database (13, 14). Using the reported structure of $ScFv_{2E8}$, the tertiary structure of $ScFv_{2E8}$-C_H1-gp41 was constructed.

pET-his-$ScFv_{2E8}$-C_H1-gp41 plasmid construction

ScFv gene was constructed by overlap extension PCR. A short peptide linker sequence $(Gly_4\text{-}Ser_1)_3$ was inserted between V_H and V_L and formed V_H-linker-V_L orientation. The C_H1 fragment derived from mAb_{2E8} was ligated at the 3'

2E8 V_H-C_H1 (part) nucleotide and deduced amino acid sequence

Leader peptide

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ATG GCT GTC CTG GGG CTG CTT CTC TGC CTG GTG ACG TTC CCA TGT GTC CTG TCC CAG GTG CAG TTG AAG
 M   A   V   L   G   L   L   L   C   L   V   T   F   P   C   V   L   S   Q   V   Q   L   K
GAG TCA GGA CCT GGC CTG GTG GCT CCC TCA CAG AGC CTG TCC ATC ACA TGC ACT GTC TCT GGA TTC TCA
 E   S   G   P   G   L   V   A   P   S   S   Q   L   S   I   T   C   T   V   S   G   F   S
                    CDR1
TTA TCA GGA TAT AGT GTA CAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG GGA ATG ATA
 L   S   G   Y   S   V   H   W   V   R   Q   P   P   G   K   G   L   E   W   L   G   M   I
                CDR2
TGG GGT GGT GGA AAC ACA GAC TAT AAA TCA GCT CTC AAA TCC AGA CTG ACC ATT AGT AAG GAC AAC TCC
 W   G   G   G   N   T   D   Y   K   S   A   L   K   S   R   L   T   I   S   K   D   N   S
AGG AGC CAA GTT CTC TTA AAA ATG AAC AGT CTG CAA ATT GAT GAC ACA GCC ATT TAC TAC TGT GCC AGA
 R   S   Q   V   L   L   K   M   N   S   L   Q   I   D   D   T   A   I   Y   Y   C   A   R
                    CDR3
AAT TAC GGA TAT AGT CCG TTT GTT CAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA
 N   Y   G   Y   S   P   F   V   H   W   G   Q   G   T   L   V   T   V   S   A
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Constant region (C_H1)

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GCC AAA ACG ACA CCC CCA TCT GTC TAT CCA CTG GCC CCT GGA TCT GCT GCC CAA ACT AAC TCC ATG GTG
 A   K   T   T   P   P   S   V   Y   P   L   A   P   G   S   A   A   Q   T   N   S   M   V
ACC CTG GGA TGC CTG GTC AAG GGC TAT TTC CCT GAG CCA GTG ACA GTG ACC TGG AAC TCT GGA TCC CTG
 T   L   G   C   L   V   K   G   Y   F   P   E   P   V   T   V   T   W   N   S   G   S   L
TCC AGC GGT GTG CAC ACC TTC CCA GCT GTC CTG CAG TCT GAC CTC TAC ACT CTG AGC AGC TCA GTG ACT
 S   S   G   V   H   T   F   P   A   V   L   Q   S   D   L   Y   T   L   S   S   S   V   T
GTC CCC TCC AGC ACC TGG CCC AGC GAG ACC GTC ACC TGC AAC GTT GCC CAC CCG GCC AGC AGC ACC AAG
 V   P   S   S   T   W   P   S   E   T   V   T   C   N   V   A   H   P   A   S   S   T   K
GTG GAC AAG AAA ATT GTG CCC AGG GAT TGT ACT AGT
 V   D   K   K   I   V   P   R   D   C   T   S
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2E8 V_L-C_L1 (part) nucleotide and deduced amino acid sequence

Leader peptide

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ATG GAT TCA CAG GCC CAG GTT CTT ATG TTA CTG CTG CTA TGG GTA TCT GGT ACT TGT GGC GAC ATT GTG
 M   D   S   Q   A   Q   V   L   M   L   L   L   L   W   V   S   G   T   C   G   D   I   V
ATG ACA CAG TCT CCA TCC TCT CTG GCT ATG TCA GTA GGA CAG AAG GTC ACT ATG AGC TGC AAG TCC AGT
 M   T   Q   S   P   S   S   L   A   M   S   V   G   Q   K   V   T   M   S   C   K   S   S
                CDR1
CAG AGC CTT TTA AAT AGT GAC AAT CAA AAG AAC TAT TTG GCC TGG TAC CAG CAG AAA CCA GGA CAG TCT
 Q   S   L   L   N   S   D   N   Q   K   N   Y   L   A   W   Y   Q   Q   K   P   G   Q   S
                            CDR2
CCT AAA CTT CTG GTG TAC TTT GCA TCC AGT AGG GAA TCG GGG GTG TCT GAT CGG TTC ATA GGC AGT GGC
 P   K   L   L   V   Y   F   A   S   S   R   E   S   G   V   S   D   R   F   I   G   S   G
TCT GGG ACA GAT TTC ACT CTT ACC ATC GGC AGT GTG CAG TCT GAA GAC CTG GCA TAT TAC TTC TGT CAG
 S   G   T   D   F   T   L   T   I   G   S   V   Q   S   E   D   L   A   Y   Y   F   C   Q
                CDR3
CAA CTT TAT AGA ACT CCA TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA
 Q   L   Y   R   T   P   F   T   F   G   S   G   T   K   L   E   I
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Constant region (C_L1)

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CGG GCT GAT GCT GCA CCA ACT GTA TCC ATC TTC CCA CCA TCC AGT GAG CAG TTA ACA TCT GGA GGT GCC
 R   A   D   A   A   P   T   V   S   I   F   P   P   S   S   E   Q   L   T   S   G   G   A
TCA GTC GTG TGC TTC TTG AAG CTT
 S   V   V   C   F   L   K   L
```

Figure 1. Nucleotide and deduced amino acid sequence of 2E8 V_H-C_H1 (part) and V_L-C_L1 (part). The nucleotide sequences for 2E8 V_H-C_H1 (part) and V_L-C_L1 (part) are shown. The leader peptide sequences are underlined and the complementary determining regions (CDRs) are shown in boxes.

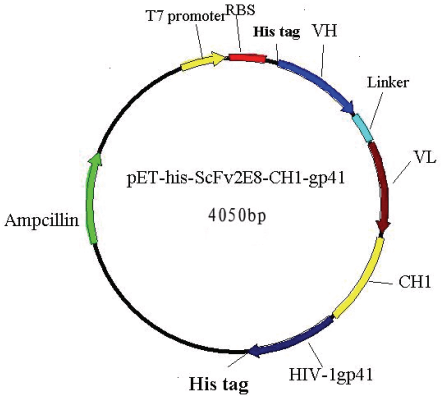

Figure 2. Schematic of plasmid pET-his-ScFv$_{2E8}$-C$_H$1-gp41. The recombinant gene ScFv$_{2E8}$-C$_H$1-gp41 is behind the T7 promoter. Before the recombinant gene, a His tag is from the pET-his vector and another His tag is added behind the recombinant gene by designer for the better affinity.

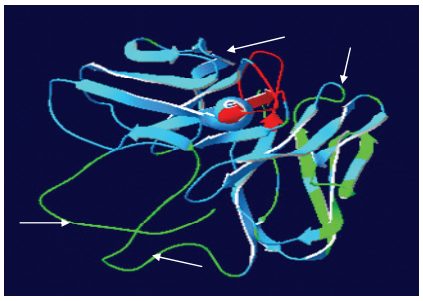

Figure 3. Prediction of tertiary structure of fusion protein from ScFv$_{2E8}$-C$_H$1-gp41 gene. The fragment of ScFv$_{2E8}$ forms typical immunoglobulin structure. Another two fragments following ScFv$_{2E8}$ are the C$_H$1 fragment and HIV-gp41 conserved region. The image was displayed by DeepView-Swiss-PdbViewer.

terminal of V_L, and the conserved region sequence of HIV gp41 (the peptide sequence of HIV-1gp41-CGT ATC CTG GCC GTC GAA CGT TAT CTG AAA GAT CAG CAG CTG CTG GGC ATC TGG GGC TGC TCT GGC AAA CTG ATC TGC ACC ACG GCG GTC CCG TGG AAT GCG TCT) was ligated by multiple PCR at the 3' terminal of C$_H$1 fragment to construct recombinant ScFv$_{2E8}$-C$_H$1-gp41 gene; and eight primers were used (Table 1). Then the recombinant gene was linked to pMD18-T vector (pMD-18T-ScFv$_{2E8}$-C$_H$1-gp41) and transformed into *E. coli* DH5α cells. Two independent clones were sequenced and compared for identity.

Expression and purification of ScFv$_{2E8}$-C$_H$1-gp41

To express the ScFv$_{2E8}$-C$_H$1-gp41 gene, the expression vector pET-his (Jingmei Biotech) was used to construct pET-his-ScFv$_{2E8}$-C$_H$1-gp41 and transformed into BL21(DE3)plysS of *E. coli*. The single colony of transformed *E. coli* was picked out and inoculated in a flask containing 20 ml of LB medium supplemented with ampicillin (150 mg/ml, 1% glucose) and cultivated at 30°C 70-90 rpm for 10 h as the seed cells. On day 2, the seed cells were added into 200 ml of LB medium supplemented with ampicillin (150 mg/ml, 1% glucose), at 37°C 300 rpm for 2 h until cell density OD$_{600}$ reached 0.6. The culture was induced by addition with IPTG at the final concentration of 100 μg/ml for 1.5 h at 30°C.

To purify fusion protein, the bacteria were harvested by centrifugation at 4,000× g for 20 min at 4°C, resuspended in 8 mol/L urea, 0.1 mol/L Na-phosphate and 10 mmol/L Tris (pH 8.0) and sonicated to reduce viscosity. The cellular debris was removed by centrifugation at 4°C for 15 min at 8,000× g. The supernatant was applied to His-bind resin column and the bound antibodies were recovered according to the manufacturer's instructions. The eluent was dialyzed against refolding buffer I (2 mmol/L reduced glutathione and 0.2 mmol/L oxidized glutathione) at 4°C for 10 h, then continued to dialyze against refolding buffer II (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.5) at 4°C for 10 h. The final product was filtrated with 0.22 μm filter membrane and stored at 4°C.

SDS-PAGE and Western blotting

For expression product analysis, the fusion protein was investigated through the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting assay was performed with the anti-His antibody after the SDS-PAGE. All steps were performed according to manufacturer's instructions. About 30 μl of sample was run on 12% sodium dodecyl sulfate polyacrylamide gel for identifying fusion protein, the product of ScFv$_{2E8}$-C$_H$1-gp41 gene. The separated proteins were transferred to nitro-cellulose filter (Millipore HAWP) using buffer (25 mmol/L Tris-HCl pH 8.0, glycine, 20% methanol). In following washing procedure between every two steps, PBST was used three times for 5 min. The membranes were first blocked for 1 h with the blocking buffer, followed by adding HRP-labeled goat anti-mouse antibody, then incubated for another 1 h, and visualized by adding the combined substrate of H_2O_2 and 4-chloro-1-naphthol.

Competitive ELISA assay

The broken membranes of RBCs were coated on each well of a 96-well plate. Renatured fusion protein (the concentration of 15-100 μg/ml) was added and incubated on the plate. After incubation, 10.5 μg/ml of mAb$_{2E8}$ was added. Unbound mAb$_{2E8}$ and fusion protein were removed by washing three times with assay buffer. An anti-mouse IgG-HRP conjugate was used to detect the bound mAb$_{2E8}$. All the incubation steps were performed for 1 h at 37°C. The reaction was developed using o-phenylenediamine (OPD) as a chromogenic substrate. Inhibition percentages were calculated as 1 - (A/B) × 100%, where A and B were the absorbance values at 492 nm for a given fusion protein concentration and no fusion protein added respectively.

Immunofluorescence

Human RBCs (7 × 10^7, type O) and 500 μl of 0.9% saline

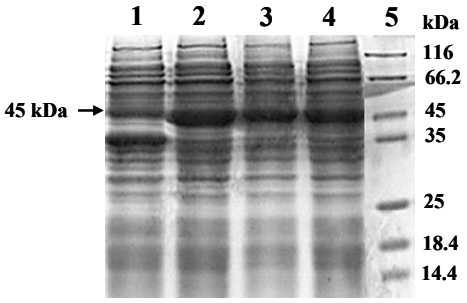

Figure 4. SDS-PAGE of fusion protein. The fusion protein was on a 12% gel and visualized by Coomassie brilliant blue staining. The arrow indicates the target band (45 kDa) in the lanes. Lane 1, whole cell lysate of BL21(DE3)pLysS with pET-his vector induced by 100 μg/ml IPTG; Lanes 2-4, whole cell lysate of BL21(DE3)pLysS with pET-his-ScFv$_{2E8}$-C$_H$1-gp41 vector, induced by 100 μg/ml IPTG; Lane 5, protein weight markers.

were mixed together at room temperature in a reaction pipe. Fifty μl of fusion protein (0.4 mg/ml) was added into the reaction pipe and mixed gently. The reaction pipe was incubated for 30 min at 37°C in all subsequent steps and RBCs were harvested by centrifugation at 3,500 rpm for 5 min at room temperature, resuspended in 500 μl of 0.9% saline twice between every two steps. Five μl of anti-His tag antibody was added to combine with the fusion protein and 5 μl of FITC-conjugated goat anti-mouse IgG antibody was added as the secondary antibody. When the RBCs combined the fusion protein with anti-His tag antibody and FITC-conjugated goat anti-mouse IgG antibody completely, 10 μl of RBCs suspension was taken and observed under fluorescence microscope (Nikon UFX-II).

Bifunctional protein for haemagglutination

Quantitative microplate agglutination assay to detect anti-HIV antibodies in 30 plasma samples was carried out.

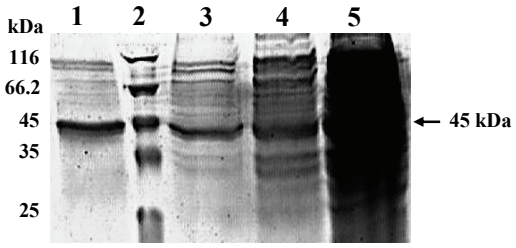

Figure 5. SDS-PAGE of purified fusion protein. The fusion protein was on a 12% gel and visualized by Coomassie brilliant blue staining. The arrow indicates the target band (45 kDa) in the lanes. Lane 1, the fusion protein obtained by the third time applying the His-bind resin column; Lane 2, the protein markers; Lane 3, the fusion protein obtained by the second time applying the His-bind resin column; Lane 4, the fusion protein obtained by the first time applying the His-bind resin column; Lane 5, the fusion protein before applying the His-bind resin column.

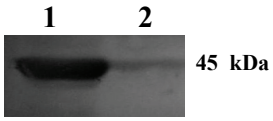

Figure 6. Western blotting analysis of bifunctional protein. Lane 1, BL21(DE3)pLysS with pET-his-ScFv$_{2E8}$-C$_H$1-gp41 vector induced with 100 μg/ml IPTG; Lane 2, BL21(DE3)pLysS with pET-his vector induced by 100 μg/ml IPTG.

Human RBCs of type O RhD-negative blood were washed twice with 0.9% saline. To 3-5% RBC suspension, purified fusion protein was added to a final concentration of 1 μg/ml. Twenty μl of RBCs and 10 μl of positive plasma and 10 μl of fusion protein were added to a microplate respectively. The microplate was incubated at room temperature for 5 min and agglutination end point was recorded.

Results

Construction of ScFv$_{2E8}$-C$_H$1-gp41 gene

5' RACE (15, 16) was used to clone the immunoglobulin (Ig) genes encoding variable regions of heavy and light chains (V$_H$ and V$_L$) from the hybridoma cell line 2E8, which secreted the monoclonal antibody (mAb$_{2E8}$) against H antigen on human erythrocytes. The V$_H$ gene consisted of 351 bp and the deduced amino acid sequence was closely related to the published sequences of the variable region I (B) subgroup of mouse Ig heavy chain. The V$_L$ gene consisted of 339 bp and the deduced amino acid sequence was highly similar to the published sequences of the variable region I subgroup of mouse Igκ light chain (17) (Figure 1).

For the construction of the bifunctional molecules, a multiple PCR was used to form the recombinant ScFv$_{2E8}$-C$_H$1-gp41 gene containing the ScFv$_{2E8}$ fragment against H antigen on RBC and a conserved region sequence of HIV gp41 and the C$_H$1 fragment derived from the constant region of the mAb$_{2E8}$. The agarose gel electrophoresis (AGE, 15 g/L) showed the PCR products in the assay of the amplification of ScFv$_{2E8}$-C$_H$1-gp41 gene was 1,150 bp, which was definitely the same size as predicted. The successful construction of the pET-his-ScFv$_{2E8}$-C$_H$1-gp41 plasmid was identified by the sequencing outcome (Figure 2). The three dimensional structure of ScFv$_{2E8}$-C$_H$1-gp41 was predicted and depicted in Figure 3.

Identification of bifunctional protein by SDS-PAGE and Western blotting

SDS-PAGE result showed that there was one more additional band in recombinant bacteria group than the control, which was just the same size (about 45 kDa) as predicted (Figure 4). To purify recombinant fusion protein, bacteria were harvested and sonicated, and the cellular debris was removed by centrifugation at 4°C. The supernatant applied to His-bind resin column and the eluent was dialyzed against refolding buffer I, then continued to dialyze against refolding buffer II.

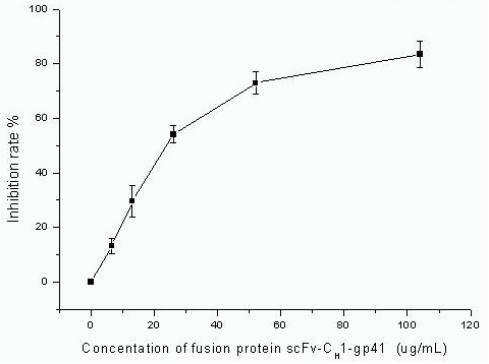

Figure 7. Inbibition rate of mAb_{2E8} binding to erythrocyte by competition with bifunctional fusion protein. Low and high marks represent mean ± standard deviation in three independent experiments.

The final product was filtrated with 0.22 μm filter membrane and stored at 4°C. After three times applying the His-bind resin column, a relatively purified protein was obtained (Figure 5).

For improving the quantity of the target protein, a pET-his vector system was used to express the bifunctional protein in the form of inclusion bodies in periplasmic space, and obtain larger amount of concentrated fusion protein (2.5-3 g/L) by BCA (bicinchoninic acid, Bradford) method.

Western blotting identified a hybridization band which was found only in recombinant bacteria lane, and was not detected in control group (Figure 6). It suggested that the expressed product of recombinant bacteria was the target fusion protein.

Functional detection of the bifunctional protein

Competitive ELISA assay was performed to identify if mAb_{2E8} and bifunctional protein bind the same epitope on the membrane of RBCs (Figure 7). The result indicated that the bifunctional fusion protein as mentioned above kept the antigenic specificity of the native antibody since its binding to H antigen was inhibited by mAb_{2E8} in a dose-dependent manner.

For visualizing demonstration of the binding of bifunctional protein with RBCs, an immunofluorescence microscopy was performed. Under the microscope the green fluorescence circling the RBC was observed and the formation of RBC was displayed. No green fluorescence was found in the control without adding anti-His tag antibody (Figure 8).

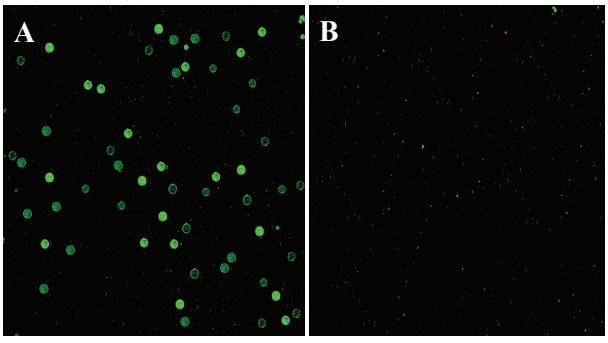

Figure 8. Immunofluorescence of bifunctional fusion protein. RBCs of blood group O were coated with fusion protein. The anti-His tag monoclonal antibody and FITC-conjugated goat-anti-mouse IgG were added separately. After washing, the cells were observed under fluorescence microscope (Nikon UFX-II). (A) RBCs with green circle. (B) Control without adding anti-His tag antibody.

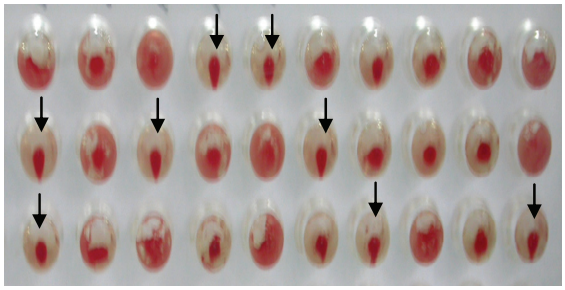

Figure 9. Mimic autologous erythrocyte agglutination of bifunctional protein with 30 plasma samples from HIV-infected individuals. The arrows indicate the weak agglutination samples.

Application of bifunctional protein in haemagglutination assay

Since fusion protein was designed for use in hemagglutination-based assay for detection of anti-HIV antibody, it was evaluated in a microtiter plate-based agglutination assay using 30 HIV positive samples (AIDS Test Center of PLA) that were confirmed by Western blotting. Twenty-two of 30 positive samples showed strong agglutination with fusion protein and 8 of 30 showed weak reactivity (Figure 9). When increasing the quantity of the weak agglutination samples in the well, agglutination became stronger. It was possible that low titer of anti-gp41 antibodies in the samples might not be sufficient to produce strong agglutination of RBCs. Thirty antibody-negative samples (from The General Hospital of PLA) showed no agglutination with bifunctional protein (data not shown).

Discussion

We had designed and constructed a bacterial expression vector to produce a fusion protein of $ScFv_{2E8}$-C_H1-gp41 bispecific molecule in *E. coli*. The fusion protein was purified to homogeneity and examined for antigen reactivity. Like the native mAb from which it was derived, the recombinant fusion protein showed binding activity and specificity for H antigen on erythrocyte. Meanwhile the fusion protein $ScFv_{2E8}$-C_H1-gp41, as the main ingredient of reagent for autologous erythrocyte agglutination test, could agglutinate RBCs in the presence of HIV-1 gp41 antibodies

using sera from HIV-infected individuals.

The development of antibody engineering technology had allowed the modification of existing mouse mAbs into recombinant segments that kept high affinity and specificity. For this purpose, one of the first things was the construction of the recombinant smallest antibody fragment with antigen binding specificity, single-chain Fv. ScFv was composed of antibody heavy and light chain variable domains ligated by a short linker peptide (18). Generally, the variable region genes of antibody were generated by PCR. Originally, the variable region gene cloning of antibody was performed by using mixtures of degenerate oligonucleotides. Although the degenerate oligonucleotide primers were designed to anneal to the conserved residues at the beginning of framework one, the use of degenerate oligonucleotides could produce sequencing mistakes because of the random mismatching (19). As for the primers designed from the leader peptide sequence of antibodies might avoid random mismatching in the conserved region, the putative primers might not be consistent with that of the wild-type gene since the signal peptide were different in the sequence of antibodies (20).

In this study, in order to make sure the integrity and accuracy of variable region, a 5' RACE method was used for the gene cloning from the mAb_{2E8} (21). In brief, the first strand cDNA were synthesized by reverse transcriptase from the total RNA, and a poly-G sequence was anchored at the 5' terminal of cDNA by terminal transferase. The variable region genes were amplified by PCR with a forward poly-C anchor primer and a reverse primer specific for constant region sequence. The amino aicd sequence of heavy and light chain and leader peptide were demonstrated to be correct by the analysis of Kabat data base.

Following the cloning of the V_H and V_L, an ScFv gene was constructed by overlap extension PCR in which the peptide linker between the V_H and V_L was encoded in the PCR primer. The flexible linker $(Gly_4Ser)_3$ was used to link the two variable region fragment (V_H and V_L) which was hydrophilic and could preclude interaction with the V-regions during folding (22). Though ScFv had a lot of advantages in the immunological assays and therapeutic use sometimes, it would not be sufficient for target use in its unconjugated form. On the contrary, the form of ScFv fusion protein would be desired molecule for application. For example, recombinant immunoglobulin fragment linked to either C terminal immuno-reactive peptide epitopes or a reported enzyme may be used as bifunctional diagnostic reagents (22).

In our study, a C_H1 fragment derived from mAb_{2E8}, as a spacer, between $ScFV_{2E8}$ and gp41 gene, was introduced at the 3' terminal of V_L and could either enhance the stability of the fusion protein or separate the functional domain of HIV-1 gp41 from that of $ScFv_{2E8}$ in the space. Then, the conserved sequences of the HIV-1 gp41 gene were ligated at the 3' terminal of C_H1 fragment by multiple PCR. So the formation of ScFv fusion protein bears two kinds of different functions that it could bind the H antigen on RBCs with the domain of ScFv and react with anti-gp41 antibodies with gp41 peptide domain in the whole blood.

The development of methods for the expression of ScFv fusion protein in *E. coli* had offered a convenient way to produce the engineering antibodies. A number of different molecules had been expressed in *E. coli* in soluble form, though the yield was lower. A relatively fine approach to high level production of ScFv fusion protein relied on cytosolic expression as inclusion bodies followed by denaturation and *in vitro* renaturation procedure.

In our study, a pET-his vector of *E. coli* was used to express our target protein ($ScFv_{2E8}$-C_H1-gp41) in the form of inclusion bodies. In order to obtain high amounts of concentrated fusion protein for purification, a strategy of denaturation and renaturation was adapted (23) and a relatively good yield (2.5-3 g/L) was got.

Although it has been reported that ScFvs with V_H-linker-V_L orientation could not be expressed (24, 25), the bi-specific molecule gene $ScFv_{2E8}$-C_H1-gp41 containing V_H-linker-V_L in the ScFv terminus of $ScFv_{2E8}$-C_H1-gp41 was also produced in the bacterial expression system.

The immunoglobulin gene fragments could be engineered to contain C terminal polypeptide tails which permits easy detection and purification of the recombinant antibodies. In the present study, a His-tag was introduced into the $ScFv_{2E8}$-C_H1-gp41 gene and formed two tags including the vector's His-tag at both the terminals of the recombinant gene, thereby facilitating the protein's recovery through nickel affinity chromatography and renaturation with GSH/GSSH system (26).

In this experiment, the positive sample had been confirmed by Western blotting. In China the HIV-1 was the majority type of HIV, and the gp41 peptide sequence was the main conserved region that triggered the immunological response in the human body. But the difference of individuals in the immune system gave rise to different titer response. The higher titer, the stronger agglutination reaction.

We successfully designed and prepared bifunctional fusion protein from inclusion bodies in *E. coli*, which had the capability of binding to both human RBCs and HIV-1 antibody. When anti-HIV-1 antibody was present in human blood specimens and the agglutination could be visualized with the naked eyes. Autologous red cell agglutination assay had the advantages of shorter detection time, simple procedure, no special equipment and direct-viewing results. The technology described in this paper could also be used to develop similar molecules for detection of various infections, such as hepatitis B, hepatitis C and syphilis, which were relevant in blood transfusion.

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