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Cloning, expression, purification, and characterization of LC-1 ScFv with GFP tag*

LU Min (卢 敏)†, GONG Xing-guo (龚兴国)‡, YU Hong (于 红), LI Jian-yong (郦剑勇)

(*Institute of Biologic Macromolecule and Enzyme Engineering, School of Life Science, Zhejiang University, Hangzhou 310027, China*)

†E-mail: sc9910@hotmail.com

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Abstract: Total RNA was isolated from the hybridoma cell line (LC-1), which secretes anti-lung adenocarcinoma monoclonal antibody, and was transferred into cDNA. Based on the FR1 (framework region 1) and FR4 conserved regions of LC-1 gene, the variable regions of heavy chain (Vh) and light chain (Vl) were amplified, and the Vh and modified Vl were connected to single chain Fv (ScFv) by SOE-PCR (splice overlap extension PCR). The modified ScFv was fused with green fluorescent protein (GFP) and introduced into *E. coli* JM109. The fusion protein induced by IPTG (Isopropylthiogalactoside) was about 57000 on a 10% SDS-PAGE gel (10% Sds Polyacrylamide Gel Electrophoresis), and primarily manifested as inclusion bodies. The renatured protein purified by Ni-NTA Superflow resins showed ability to bind to antigen on SPC-A-1 lung adenocarcinoma. In addition, the induced host cells fluoresced bright green under 395 nm wavelength, which indicated that the expected protein with dual activity was expressed in the prokaryotic system. The ScFv with GFP tag used in this research can be applied as a new reagent to detect immunological dye, and provide a feasible way to detect adenocarcinoma in a clinical setting.

Key words: ScFv (single chain variable fragment), GFP (green fluorescent protein) tag, Protein fusion, Purification
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INTRODUCTION

Because of its low molecular weight and ability to fluoresce independently (George, 1997), the new molecular tag, green fluorescent protein (GFP), has become more and more popular after Prasher *et al*.(1992) cloned its cDNA in 1992. There are many reports describing the co-expression of GFP and a specific antibody or cytokine gene, with the fusion protein expressing the fluorescent activity and biological activity of the complement protein (Haraguchi *et al*., 1999; Mclean *et al*., 1999; Walker *et al*., 1999; Otsuki *et al*., 1999; Zhu *et al*., 1999). This fusion protein can be used for tumor assessment, drug screening, and localization of cell receptors. Cheng *et al*.(2001) achieved the co-expression of GFP and anti-liver cancer ScFv. But until now, there is no report describing co-expression of GFP and anti-lung adenocarcinoma ScFv. In the study described here, LC-1 ScFv was constructed and linked with GFP for co-expression. Theoretically, the expected protein should possess dual activity so that it can be used as a new reagent to detect immunological dye and provide a feasible way to detect adenocarcinoma in clinical setting.

MATERIALS AND METHODS

Materials

Total RNA of the LC-1 hybridoma and the pProEx HTb vector were kindly provided by Dr. Chen Liang of Yale University. The pAVA319 vector was provided by Dr. Arnim. The pUCm-T vector, *E. coli* JM109, DH5α, and lung adenocarcinoma cell line are available in our institute.

The heavy chain primers were Vh-1 (AGGTC

‡ Corresponding author

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CAACTGCAGGAGTCAGG *Pst* I) and Vh-2 (TGAGGAGACGGTGACCGTGGTCCCTTGGCC CAG *Bst*E II); the light chain primers were Vl-1 (GACATTGAGCTCACCCAGTCTCCA *Sac* I) and Vl-2 (GTTTGATCTCGAGCTTGGTCCC *Xho* I); the linker for Vh and Vl was link-1 (CTGGGGCCAA GGGACCACGGTCACCGTCTCCTCAGGTGGAG GCGGTTCAGGCGGAGGTGCTCTGGCGGTGGC GGATCGGACATTGAGCTCACCCAGTCTC); and the upstream primer for modifying ScFv was ScFv-1 (GAGCTAGATCTAAGGTCCAACTGCAG *Bgl* II).

Construction of ScFv

Vh and Vl were amplified from the cDNA of the LC-1 hybridoma using RT-PCR; we used primers for Vh-1, Vh-2, and Vl-1, Vl-2, respectively. They were cloned into a pUCm-T vector, respectively. The Vl was modified to be Vl′, primered with link-1 and Vl-2, and linked with Vh through SOE-PCR. Primered with ScFv-1 and Vl-2, the *Bgl* II and *Xho* I sites were added to the resultant ScFv′, which also was cloned into the pUCm-T vector. The recombinant plasmid, called T-ScFv, was transformed into competent *E. coli* DH5α using the previously described $CaCl_2$ method (Joseph and David, 2001) and sequenced.

Construction of Pro319

The GFP was cloned into pProEx HTb by digesting the pAVA319 with *Nco* I and *Xba* I. The resultant Pro319 was transformed into JM109.

Construction of ProGFP-ScFv

The ScFv was cloned into Pro319 by digesting T-ScFv with *Bgl* II and *Xho* I. The recombinant plasmid, called ProGFP-ScFv, was transformed into JM109 (Fig.1).

Expression of GFP-ScFv fusion protein

A positive cell clone was inoculated onto 500 ml LB (50 μg/ml Amp) including HEPES buffer (pH 6.0~6.5) and incubated until OD_{60}=1.0 (Shi and Su, 2000). LB without HEPES buffer served as the negative control. The cells that were induced in the presence of IPTG gradient concentrations (0.1, 0.5, and 1 mmol/L) at 24 °C for 5 h were collected. Expressed fusion protein was analyzed on a 10% SDS-PAGE gel.

Purification of the fusion protein

Sedimentation was achieved by disrupting the induced cells using sonication (500 W, 2 min×5), and washing with wash buffer I (1 mmol/L EDTA, 5% glycerol, 0.5% Triton X-100) and buffer II (wash buffer I with 2 mol/L carbamide). Then the sedimented material was dissolved in lysis buffer (50 mmol/L Tris-HCl, 0.3% Sodium lauryl sulfate, 0.1 mmol/L Dithiothreitol) overnight. The fusion protein was renatured in lysis buffer without SLS. Finally, the renatured protein was purified by Ni-NTA Superflow resins; elution was carried out in the presence of an imidazole gradient concentration (10~150 mmol/L) and was collected per millitre, and A_{280} was measured. The eluted protein was analyzed on a 10% SDS-PAGE gel.

Competitive ELISA assay of fusion protein

The SPC-A-1 cells were coated onto each well of a 96-well plate at a density of 5×10^5 cells/well. The 0.01 μg/μl LC-1 antibody and the purified fusion protein of the gradient concentrations (original liquid was about 0.1 μg/μl, 0.01 μg/μl, or 0.001 μg/μl) were used to bind cells; goat anti-mouse labelled anti-IgM secondary antibody was used to bind to the LC-1 antibody. We read signal at A_{490} using an auto-

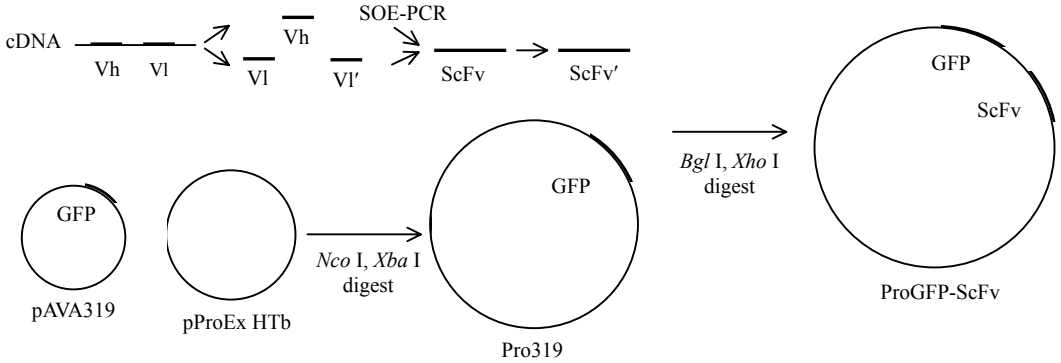

Fig.1 Construction of ProGFP-ScFv plasmid

matic reader. At the same time, the LC-1 antibody (0.01 μg/μl, 0.001 μg/μl, or 0.0001 μg/μl) and PBS served as the positive control and negative control, respectively.

Assay of fluorescent activity

The fluorescent activity of induced cells was assayed at 395 nm by fluorescent microscope (640X); cells that had not been induced served as the negative control.

Prediction of tertiary structure

The ScFv sequence was submitted to SWISS-MODEL (http://swissmodel.expasy.org/) (Schwede *et al*., 2003; Guex and Peitsch, 1997; Peitsch, 1995) by ExPASy; templates were obtained by searching the ExNRL-3D database. Using the reported structure of GFP, the tertiary structure of GFP-ScFV was constructed.

RESULTS

Construction of ScFv′

Following Orlandi *et al*.(1989) regarding FR1 and FR4 conserved regions, the primers Vh-1/Vh-2, and Vl-1/Vl-2 were designed. The final ScFv′ was about 700 bp, as expected.

Construction of ProGFP-ScFv

We obtained the expected bands (Fig.2) after digesting ProGFP-ScFv with *Bgl* II/*Xho* I and *Nco* I/*Bgl* II. The sequence of ProGFP-ScFv showed that the target plasmid had been constructed successfully, and that the ScFv and GFP were within the correct reading frame (Fig.3).

Expression and purification of GFP-ScFv

It was reported (Ogawa *et al*., 1995) that GFP is very likely to form chromophore at a low temperature. So, the expression was induced by IPTG at 24 °C. A 10% SDS-PAGE gel showed a band of about 57000, which was in accordance with the theoretical molecular weight of GFP-ScFv. In addition, the protein was primarily identified in inclusion bodies. When the expression was induced by 0.5 mmol/L IPTG, the fusion protein was composed of approximately 40% of total bacterial protein (Fig.4a).

The Ni-NTA elution (Fig.5) showed two clear peaks. The 10% SDS-PAGE gel of these peaks showed that the fusion protein was comparatively pure

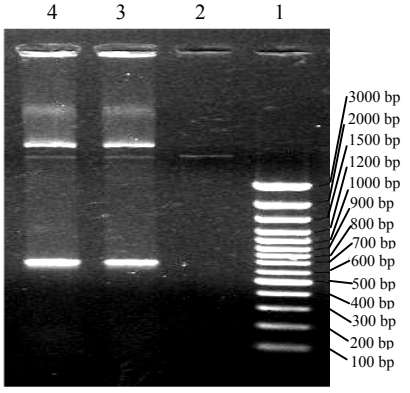

Fig.2 Digesting result of ProGFP-ScFv in 1% agrose gel. 1: Marker; 2: Nothing; 3:Digesting result of ProGFP-ScFv by *Bgl* II and *Xho* I, 4: Digesting result of ProGFP-ScFv by *Nco* I and *Bgl* II

GFP* aga tct aag gtc caa ctg cag gag tca gga cct ggc ctg gtg aaa cct tct cag tct ctg tcc ctc acc tgc act gtc act ggc tac tca atc
GFP* R S K V Q L Q E S G P G L V K P S Q S L S L T C T V T G Y S I
acc agt **gat tat gcc tgg aac** tgg atc cgg cag ttt cca gga aac aaa ctg gag tgg atg ggc **tac ata agc tac agt ggt agc act agc**
T S **D Y A W N** W I R Q F P G N K L E W M G **Y I S Y S G S T S**
tac aac cca tct ctc aaa agt cga atc tct atc act cga gac aca tcc cag aac cag ttc ttc ctg cag ttg aat tct gtg act act gag gac
Y N P S L K S R I S I T R D T S Q N Q F F L Q L N S V T T E D
aca gcc aca tat tac tgt gca aga **tct act atg att acc aca aga agg gtc ggc tac** tgg ggc caa ggg acc acg gtc acc gtc tcc tca
T A T Y Y C A R **S T M I T T R R V G Y** W G Q G T T V T V S S
ggt gga ggc ggt tca ggc gga ggt ggc tct ggc ggt ggc gga tcg gac att gag ctc acc cag tct cca gca atc atg tct gca tct cta
G G G G S G G G G S G G G G S D I E L T Q S P A I M S A S L
ggg gaa cgg gtc acc atg acc **tgc act gcc agc tca agt gta agt tcc agt tac ttg cac** tgg tac cag cag aag cca gga tcc tcc ccc
G E R V T M T **C T A S S S V S S S Y L H** W Y Q Q K P G S S P
aaa ctc tgg att **tat agc aca tcc aac ctg gct tct** gga gtc cca gct cgc ttc agt ggc agt ggg tct ggg acc tct tac tct ctc aca atc
K L W I **Y S T S N L A S** G V P A R F S G S G S G T S Y S L T I
agc agc atg gag gct gaa gat gct gcc act tat tac tgc **cac cag tat cat cgt tcc cca** ccc acg ttc gga ggg ggg acc aag ctc gag
S S M E A E D A A T Y Y C **H Q Y H R S P** P T F G G G T K L E

Fig.3 Nucleotide and deduced amino acid sequence of GFP-ScFv
The bold words represents the hypervariable regions of ScFv

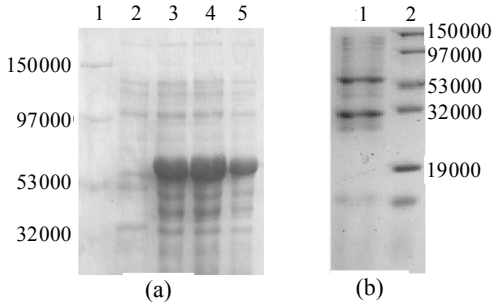

Fig.4 10% SDS-PAGE gel of GFP-ScFv fusion protein (a) Expression result of ProGFP-ScFv vector. 1: Marker; 2: Negative control; 3, 4, 5: Cell induced in different [IPTG] (0.1, 0.5, 1 mmol/L) (b) Purified GFP-ScFv in 10% SDS-PAGE gel. 1: Purified GFP-ScFv; 2: Marker

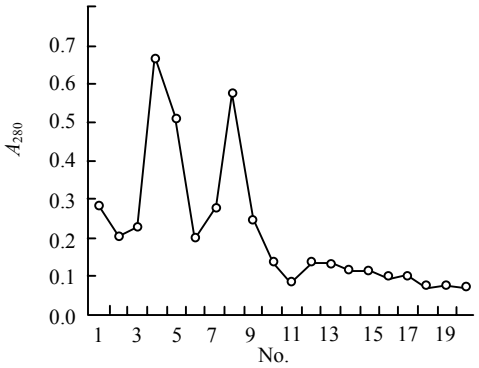

Fig.5 The elution curve for protein purified by Ni-NTA

(Fig.4b). In addition, the molecular weight of peak 2 was in accordance with what was anticipated for the protein (about 57000), while the molecular weight of peak 1 was close to that anticipated for GFP (about 28000). It is possible that the fusion protein had degraded automatically and released GFP.

Competitive ELISA assay of the fusion protein

Fig.6 shows that the 0.01 μg/μl LC-1 antibody had relatively strong ability to bind to lung adenocarcinoma (A_{490}=1.254±0.02); so, this concentration of LC-1 was used in our competitive ELISA. According to the formula: $IR(\%)=(1-A_{\text{sample}}/A_{\text{positive control}})\times 100\%$, the IR of the fusion protein with the high concentration (0.1 μg/μl, A_{490}=0.462±0.01) was 63%. It indicated that numerous epitope sites on lung adenocarcinoma were bound to the fusion protein.

Assay of fluorescent activity

Though the secreted fusion protein was small, the cells induced by IPTG fluoresced bright green under 640X fluorescent microscope (Fig.7), while the

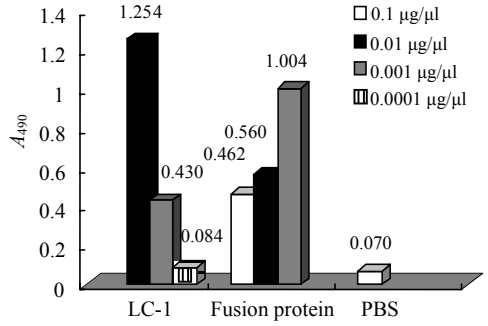

Fig.6 The ELISA curve of GFP-ScFv in A_{490}

negative control did not.

There was an interesting observation in our findings: when cells were induced in LB without HEPES for 5 h, the final pH of LB decreased to 5.1. In addition, the cells expressed much less protein (accounting for about 8% of total bacterial protein, primarily as inclusion bodies) and hardly fluoresced at 395 nm. This finding indicated the necessity for invariable pH in this expression system.

Prediction of tertiary structure

The tertiary structure of GFP-ScFv was determined as depicted in Fig.8.

CONCLUSION

Usually, the antibody gene and another gene with some biological activity are co-expressed, and the resultant molecule can be used for clinical evaluation and gene therapy (Myers *et al*., 2002; David *et al*., 2000; Mccall *et al*., 1999). In theory, the main benefits of our GFP-ScFv as opposed to conventional immunostaining methods are that this approach is rapid, purification is not essential, and it is relatively inexpensive. Also, the GFP tag is capable of eliminating background resulting from nonspecific binding of primary and secondary antibodies to targets other than the antigen, which is often a problem in immunostaining (Casey *et al*., 2000).

To preserve the antibody activity of ScFv, we cloned the integrated variable regions, including all six hypervariable regions (Fig.3). The ScFv eliminates the invariable regions so that ScFv has much lower immune binding than does McAb (monoclonal antibody) (Tina *et al*., 2004; Gao *et al*., 2003). The

Our future work will focus on application: in the clinical setting, the ScFv with a GFP tag will be used as a new reagent to detect SPC-A-1 adenocarcinoma; in an applied assay, it will be used to mark directly the specific antigen-antibody reaction.

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