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An Engineered Bivalent Single-Chain Antibody Fragment That Increases Antigen Binding Activity[1]

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Bivalent single chain Fv (scFv) was constructed by fusing a polypeptide extension containing one or two cysteines to the COOH-terminus of an scFv antibody fragment. The scFv protein was expressed and secreted in a recombinant *Pichia pastoris* system as a dimer with a C-terminal disulfide bridge, as determined by Western blot analysis under non-reducing conditions. We found that the scFv construct with one cysteine in the C-extension (scFv-1Cys) exhibited a much higher dimer/monomer ratio than the two cysteine counterpart (scFv-2Cys). Binding activity measurements performed by means of a competitive radioimmunoassay showed that scFv-1Cys exhibited specific antigen binding activity, which was almost the same as that of the parental MAb, and approximately four- and fortyfold higher than those of the control scFv monomer and scFv-2Cys.

Key words: antibody engineering, bivalent scFv, *Pichia pastoris*, single-chain Fv.

The development of murine monoclonal antibodies (MAb) for the treatment of human disease has been very successful, including for the diagnosis and therapy of cancer (*1*). An antibody fragment of single-chain Fv has been genetically engineered as a recombinant fusion protein, which is composed of heavy-chain (Vh) and light-chain (Vl) variable domains connected by an artificial linker (*2, 3*). This small molecule has shown increased accessibility to tumor cells *in vivo* (*4, 5*). However, the considerable decrease in the binding activity of scFv, compared to that of MAb, may be due to a loss of avidity of the scFv. Our previous study on antibody engineering demonstrated that the introduction of an intra-molecular disulfide linkage into the framework region enhanced the stability of scFv but had no effect on the binding activity (*6*). To obtain optimal tumor targeting and biological properties, antibody fragments with intermediate molecular weights can be designed with a proper combination of high tumor uptake and rapid clearance from normal tissues. It might be a good idea to generate a dimerized scFv by connecting two scFvs through an additional peptide linker (*7*), or through chemical cross-linking (*8*). Others have fused scFv to protein domains capable of dimerization, *e.g.*, leucine zippers (*9*), amphipathic helices (*10*), the *k* constant domain, or C_H3 in the form of a minibody (*11*), to constract non-covalent dimers. Recent biodistribution studies demonstrated that scFv in a non-covalent dimer form shows promising results as to improved targeting, *i.e.* as compared to the monomer (*12*).

Of particular interest to us was the design and expression of scFv proteins with C-terminal extensions containing a number of cysteines in order to form inter-molecular disulfide bridges. Therefore, a scFv dimer with a covalent bond might increase tumor targeting as a result of a combination of factors, including an increase in avidity of is bivalent molecule and/or a longer biological half-life due to the higher molecular weight. The strategies used to produce recombinant scFv proteins have been based on the methylotrophic yeast, *Pichia pastoris*, expression/secretion system, which was developed for the overproduction of a variety of eukaryotic proteins, particularly highly disulfide-bonded proteins (*13*), with high secretion-efficiency (reviewed in Ref. *14*). The expressed proteins with disulfide bonds, either *via* an intra- or inter-molecular interaction, can be folded correctly in the yeast *P. pastoris* system. This advantage eliminates the additional step of unfolding/refolding *in vitro* of the proteins produced in a bacterial, *e.g. Escherichia coli*, expression system.

The goal of this study was to produce scFv (monomer and dimer) derivatives in the *P. pastoris* expression/secretion system using AltaRex's cancer-therapeutic monoclonal antibody MAb B43.13, which selectively recognizes a unique epitope of CA125 expressed on ovarian carcinomas (*15*). The MAb B43.13 variable domain sequences were PCR-amplified using sequence specific primers (*16*), and engineered into a cloning vector with an scFv orientation of Vl-linker-Vh. The DNA fragment coding for the scFv was then subcloned into *P. pastoris* vector, pPIC-9, with αF secretion signals, resulting in recombinant plasmid pPIC-B43.13 (Fig. 1). Three derivatives of pPIC-B43.13, with an additional C-terminal extension containing one or two cysteines (pDL-10 and pDL-9), or a control sequence without a cysteine (pDL-11), were designed. To construct plasmids pDL-9, pDL-10, and pDL-11, DNA oligodeoxyribonucleotides (5′-GAATTCTGAGCAGAAGCTCATCT-

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Abbreviations: AOX, alcohol oxidase; CDR, complementary determining region, CPM, counts per minute; MAb, monoclonal antibody; Fv, variable fragment; PBS, phosphate-buffered saline; RIA, radioimmunoassay; scFv, single-chain Fv; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

CGGAAGAGGACCTGGGAGGACCATGCCATCCGCAG-TTCCCACGATGTTATGC-3′,5′-GAATTCAGCTGGAGG-TGGTGGATGTGC-3′, and 5′-GAATTCTGGAGGTGGT-ACCAAGCCTAGGTAGC-3′) coding for the amino acid sequences, N-ProCysHisProGlnPheProArgCysTyrAla-C, N-SerAlaGlyGlyGlyGlyCysAla-C, and N-SerGlyGlyGly-ThrLysProArg-C, were used by inserting the fragments into the *Eco*RI and *Eag*I sites of pPIC-B43 (Fig. 1).

The plasmid DNAs were transformed into competent GS115 cells by electroporation and the resulting transformants were selected on histidine-deficient medium. After screening for integration at the correct loci (*i.e.* colonies can grow on a −his/+glycerol plate but only slowly on a −his/+methanol plate), all positive clones obtained (Table I) were isolated, cultured in induction medium, and then analyzed for protein expression by SDS-PAGE followed by Coomassie staining (Fig. 2). Because the purification of recombinant proteins from host cells and medium components is a major concern at both the research and manufacturing levels, and complex biological media necessitate expensive multi-step purification processes in particular, we examined the expression and secretion of scFv in minimal and chemically defined media to overcome some of these problems. The chemical defined medium contained 10 g of yeast extract and 20 g peptone per liter, supplemented with 100 ml of 1 M potassium phosphate buffer (pH 6.0), 100 ml of 13.4% (w/v) yeast nitrogen base, 2 ml of 0.02% (w/v) biotin, and 100 ml of 5% (v/v) methanol. The composition of the minimal medium was the same as that of the chemical defined medium but with both yeast extract and peptone. We found that the same level of protein expression/secretion could be achieved, as determined by SDS-PAGE (Fig. 2, lanes 1 and 3). Direct quantitation by protein assaying using the purified material indicated that a yield of 100 mg/liter was obtained.

Because a sulfhydryl group of the C-terminal cysteine residue was introduced into scFv, site-specific dimerization of scFv through a disulfide bond might be possible. To allow

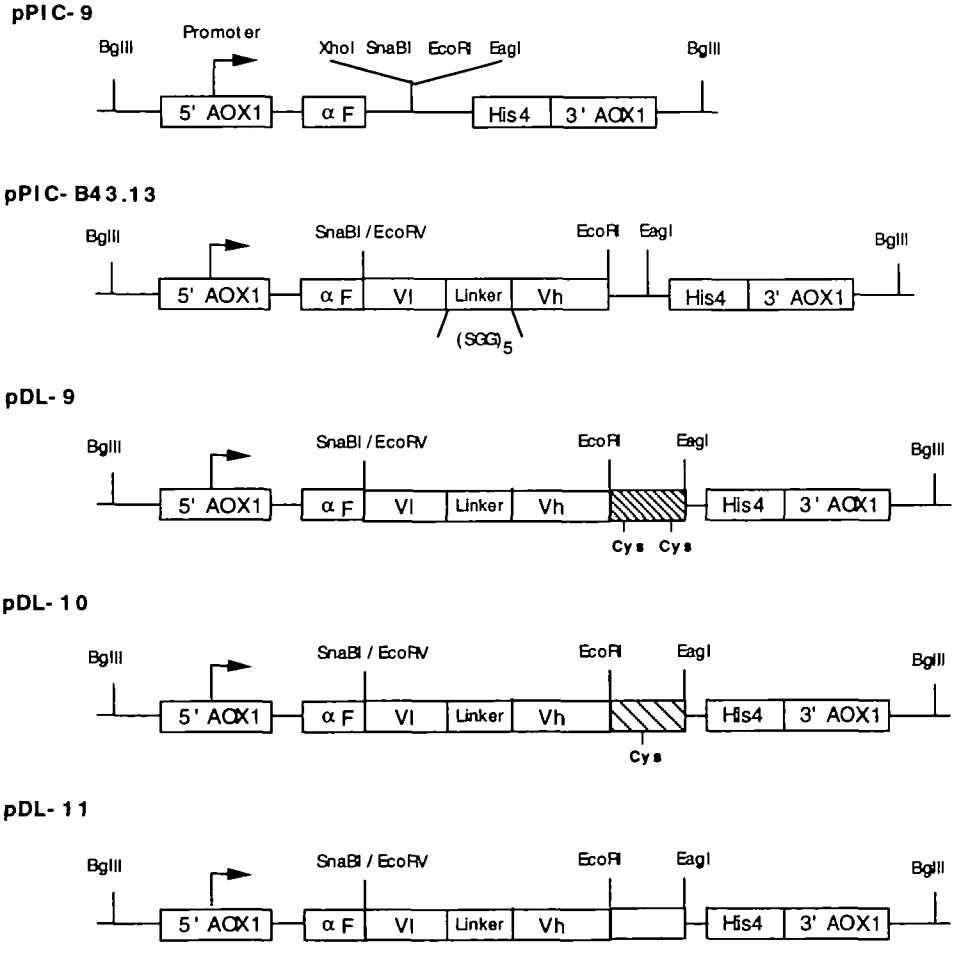

Fig. 1. **Plasmid construction of scFv-B43.13 derivatives controlled by the inducible AOX1 gene promoter.** The plasmids derived from vector pPIC-9 contained an αF secretion signal. Plasmids pDL-9, pDL-10, and pDL-11 were constructed for the expression of scFvs with different C-terminal extensions, *i.e.* containing two, one, or no cysteine.

TABLE I.

Plasmid name	Construct	Total number of His$^+$ transformants	Total number of transformants picked	Number of His$^+$, Mut$^-$ transformants	RIA[b]	Protein expression[c]
pDL-9	scFv-2Cys	>300	100	18(18%)[a]	+	+
pDL-10	scFv-1Cys	>300	100	19(18%)	+	+
pDL-11	scFv-control	>300	100	18(18%)	+	+

[a]Percentage of His$^+$ Mut$^-$ (methanol utilization deficient) tranformsants. [b]Radioimmunoassay. [c]Determined by Coomassie staining and Western blotting.

comparison of the levels of dimerization among the scFv proteins, *i.e.* scFv-2Cys and scFv-1Cys, isolated from two constructs, pDL-9 and pDL-10, and a control monomeric scFv isolated from construct pDL-11, protein samples were subjected to SDS under non-reducing conditions, and then determined by Coomassie staining (data not shown) and Western blotting (Fig. 3). In this "sandwich" assay system, another anti-CA125 murine antibody, B27.1 (at a concentration of 500 ng/ml), which reacts with a different epitope on the CA125 antigen than the one reactive with B43.13 derived scFv, was used along with antigen CA125 (at a concentration of 4,000 units/ml) as a bridge. The reactive proteins were detected using horseradish peroxidase-conjugated anti-mouse IgG, as described (*6*). The pDL-10 construct (scFv-1Cys) gave two bands corresponding to molecular masses of 25 and 50 kDa, representing the monomer and dimer of scFv in an approximate ratio of 3 to 1 (Fig. 3). However, under the same conditions, the failure to detect the scFv dimer with scFv-2Cys was a surprise. One explanation is that protein-folding *via* intra-molecular disulfide bonding might be more favorable than that *via* inter-molecular bonding, through which dimeric molecules are formed. When the proteins were concentrated about ten times and then applied to non-reducing gels, some dimer formation by scFv-2Cys in a dimer/monomer ratio of approximately 1 to 20 was observed, whereas the ratio remained very constant in the case of scFv-1Cys at the two different protein concentrations (Fig. 3, lanes 1 and 2).

It has generally been believed that recombinant proteins expressed in and secreted by recombinant *P. pastoris* cells retain their biological activities. In order to demonstrate that the scFv proteins retained their intrinsic binding activities, competitive radioimmunoassay (RIA) experiments were carried out, in which 800 units of CA125 was coated on a solid phase (200 μl of 4,000 units/ml of CA125) and 5 ng of ^{125}I labeled MAb B43.13 (100 μl of 50 ng/ml) was challenged with each scFv-containing sample. We found that the purified scFv samples isolated from pDL-9, pDL-10, and pDL11-transformed cells showed positive binding activity on RIA (Fig. 4). Furthermore, on comparison of the dimer-containing sample of scFv-1Cys with those of scFv-2Cys and the monomer control, with low to high protein concentrations, similar patterns of antigen binding were observed (Fig. 4). All three scFv derivatives showed lower binding activity than that of whole antibody MAb-B43.13. It is expected that this result might be due to the combined effects of the binding avidity and protein stability. To achieve 50% inhibition in the RIA, scFv-2Cys (pDL-9), scFv-1Cys (pDL-10), and the monomer control (pDL-11) were needed at concentrations of 3,467, 89, and

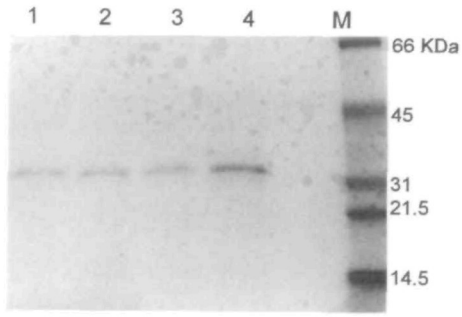

Fig. 2. **Expression of the scFv proteins in transformed *P. pastoris* cells.** Protein samples prepared from cell supernatants of representative clones were analyzed on 10% SDS-protein gels, followed by Coomassie staining. For induction of the scFv proteins in recombinant *P. pastoris* cells, two different induction media were used. Lanes 1 and 2: scFv-1Cys and scFv-2Cys produced in the chemically defined medium. Lane 3: scFv-1Cys in the minimal medium. Lane 4: Tenfold concentrated protein sample of scFv-1Cys produced in the minimal medium.

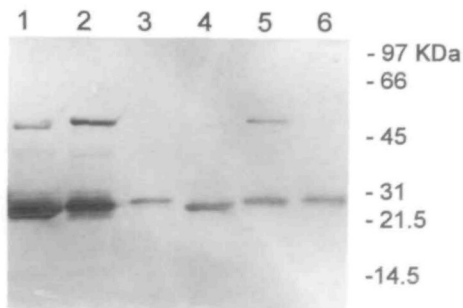

Fig. 3. **Analysis of the scFv (dimer and monomer) proteins on non-reducing SDS-gels, followed by Western blotting.** Lanes 1 and 2: Tenfold concentrated samples of scFv-2Cys (pDL-9) and scFv-2Cys (pDL-10). Lane 3: scFv-B43.13. Lanes 4 to 6: scFv-2Cys, scFv-1Cys, and scFv monomer control (pDL-11). The molecular masses of the scFv monomer and dimer were shown to be around 25 and 50 kDa, respectively.

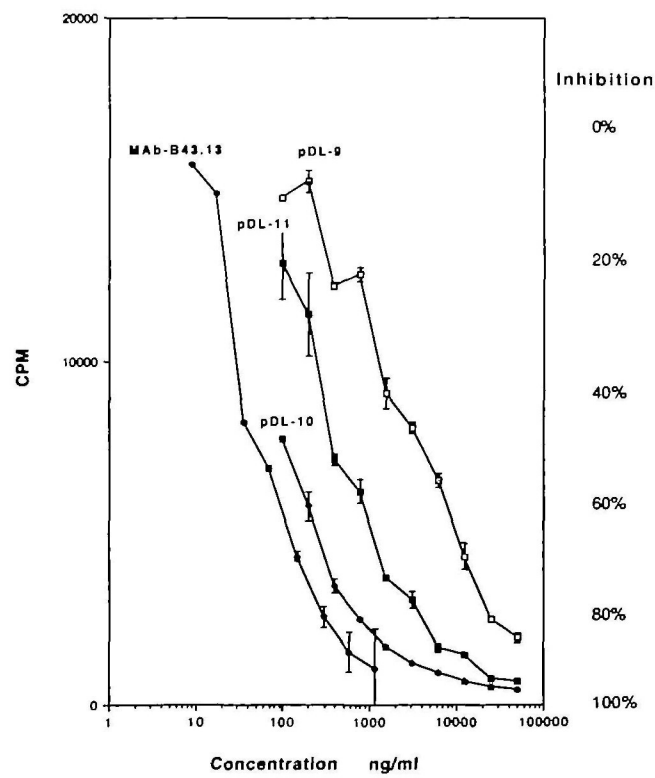

Fig. 4. **Antigen binding activity analyzed by RIA.** CA125 was coated on a solid phase, and then ^{125}I-labeled MAb-B43.13 was challenged with the scFv derivatives, *i.e.* scFv-2Cys (pDL-9), scFv-1Cys (pDL-10), and the monomer control (pDL-11), in comparison with the whole MAb-B43.13. The protein concentrations used in this assay are indicated on the *x*-axis, and the cpm corresponding to the percentage of inhibitory activity are shown on the *y*-axis.

355 ng/ml, whereas MAb-B43.13 was needed at 43 ng/ml in the same assay system. scFv-1Cys exhibited the highest antigen binding activity among the three scFv derivatives, it being approximately four and forty times higher than those of the monomer control and scFv-2Cys, although the dimeric form only accounted for about 25% of the total scFv-1Cys protein content, as estimated on the non-reducing SDS-PAGE shown in Fig. 3. It is interesting to note that such a small amount of dimeric scFv in a sample can dramatically enhance the binding activity in comparsion to the monomer control.

The binding activity of scFv-2Cys, determined by the competitive RIA (Fig. 4), was much lower than that of the scFv monomer, even though scFv-2Cys was found to be a monomer under non-reducing conditions, as shown in Fig. 3. The exact reason for the decrease in the binding activity is unknown. It is most likely that the C-terminal extension of scFv-2Cys interferes with the overall binding conformation of the scFv, particularly the CDR finger structures. The original idea was to include an epitope sequence between the two cysteines in the scFv-2Cys construct, for rapid detection. The loop structure in the C-extension might be formed through a disulfide bridge between the two C-terminal cysteines (six amino acids in the loop), as we recently obtained a similar result in a phage library presentation experiment (unpublished data). Although any loop structures may contribute to some non-specific binding, it is our opinion that the generation of a C-terminal loop might be a good reason for the decrease in the specific binding. Therefore, it might be important for future engineering to avoid the creation of any loop structure in the C-extension by bringing the two cysteines much closer to each other with only one or two amino acids between them.

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