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Construction and Expression of Bi-Functional Proteins of Single-Chain Fv with Effector Domains[1]

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We fused various polypeptide extensions to the C-termini of single chain Fv (scFv) and disulfide-stabilized Fv (dsFv) fragments to facilitate detection of bi-functional proteins or to add biological effector domains, which included the human metallothionein (HMT) motif and biotin mimetic sequence. These bi-functional proteins were expressed and secreted in a recombinant *Pichia pastoris* system and showed specific anti-idiotype binding activity, as determined by competitive radioimmunoassaying. However, the fusion protein constructed with dsFv- HMT, but not scFv-HMT, had lost this binding activity. The interruption of the structural conformation as a result in dsFv-HMT may be explained by the interactions between the cysteines engineered in dsFv domains and the cysteines in the HMT region.

Key words: antibody engineering, bi-functional, disulfide bond, *Pichia pastoris*, single-chain Fv.

The use of murine monoclonal antibodies (MAb) for the treatment of human diseases has been very successful, including in the diagnosis and therapy of cancer (*1*). Single-chain Fv has been genetically engineered as a recombinant fusion protein composed of a heavy-chain (Vh) and a light-chain (Vl) variable domain connected by an artificial linker (*2, 3*). This small molecule shows increased accessibility to tumor cells *in vivo*, and may therefore be better than MAb for use in drug, radionuclide or hormone delivery systems (*4, 5*).

Of particular interest to us is the design and expression of small bi-functional scFv proteins for use in tumor cell imaging for cancer diagnosis, a representative structure model (scFv-174-HMT) being shown in Fig. 1. The strategies previously used to produce bi-functional scFv proteins were based on a methylotrophic yeast, *Pichia pastoris*, expression/secretion system which was developed for the overproduction of a variety of eukaryotic proteins with high secretion-efficiency (reviewed in Ref. *6*). In this system, the DNA fragment coding for scFv and the functional motif is inserted in the place of the *P. pastoris* alcohol oxidase (AOX1) gene, and thus the expression of the cloned gene is under the control of the strong and methanol inducible AOX1 gene promoter.

The goal of this study was to overproduce bispecific functional scFv/dsFv in the *P. pastoris* expression/secretion system using Biomira's cancer-imaging monoclonal antibody, MAb 174H.64 (Tru-Scint^{R}SQ™ kit, Biomira), which selectively recognizes a unique epitope expressed on proliferating squamous carcinomas (*7, 8*). The DNA frag-

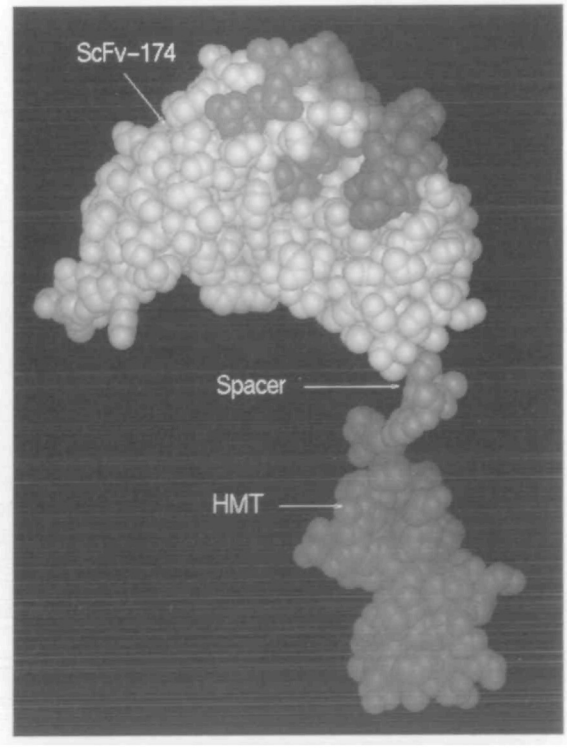

Fig. 1. A three-dimensional model of the bi-functional protein of a scFv fragment with a HMT domain connected by a spacer.

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Abbreviations: AOX, alcohol oxidase; CDR, complementary determining region; MAb, monoclonal antibody; Fv, variable fragment; HMT, human metallothionein; PBS, phosphate-buffered saline; RIA, radioimmunoassay; scFv and dsFv, single-chain and disulfide-stabilized Fv; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

ments coding for the engineered scFv-174 and dsFv-174 with an additional functional domain at the C-terminus (HMT domain or biotin mimetic sequence) were separately constructed in the *P. pastoris* vector, pPIC-9, with aF secretion signals, resulting in four recombinant plasmids (Fig. 2). The functional domain containing the human metallothionein (HMT) or KCTCCA sequence can be used as a radioactive metal-binding domain (*9*), and the biotin mimetic sequence can be used for the interaction of fluorescence-conjugated or radioactive labeled streptavidin for tumor imaging and therapy. Metallothionein is a cysteine-rich protein that binds to many metals, and has particularly strong affinity for mercury, cadmium, and technetium (*10*), and hexapeptide KCTCCA, a short version of HMT, derived from the C-terminus of the metallothionein sequence has been examined as to binding to cadmium and technetium (*11, 12*), and was found to function well (*12*). Therefore, the HMT or KCTCCA domain was used in this study as a functional domain, *e.g.* as a chelator for technetium.

The construction of plasmids pBM045 (scFv-174-HMT) and pBM046 (dsFv-174-HMT) was described previously (*13*). To construct pBM076 and pBM084, DNA oligodeoxyribonucleotides (5′-GAATTCTGAGCAGAAGCTCATCTCGGAAGAGGACCTGGGAGGACCATGCCATCCGCAGTTCCCACGATGTTATGCGGCC-3′ and 5′-GAATTCTGGAGGTAAGTGTACATGTTGCGCATGTTGCGCGGCC-3′) coding for the biotin mimetic sequence (N-ProCysHisProGlnPheProArgCysTyrAla) and the KCTCCA sequence (N-LysCysThrCysCysAlaCysCysAla) were used by replacing HMT fragments in pBM045 using *Eco*RI and *Eag*I sites (Fig. 2). The spacer sequences (spacer A: SerGluGlnLysLeuIleSerGluGluAspLeuGlyGly; spacer B: SerGlyGly) between the two functional domains were added according to Cwirla *et al.* (*14*).

The plasmid DNAs were transformed into competent GS115 cells by electroporation and the resulting transformants were selected on histidine-deficient medium. After

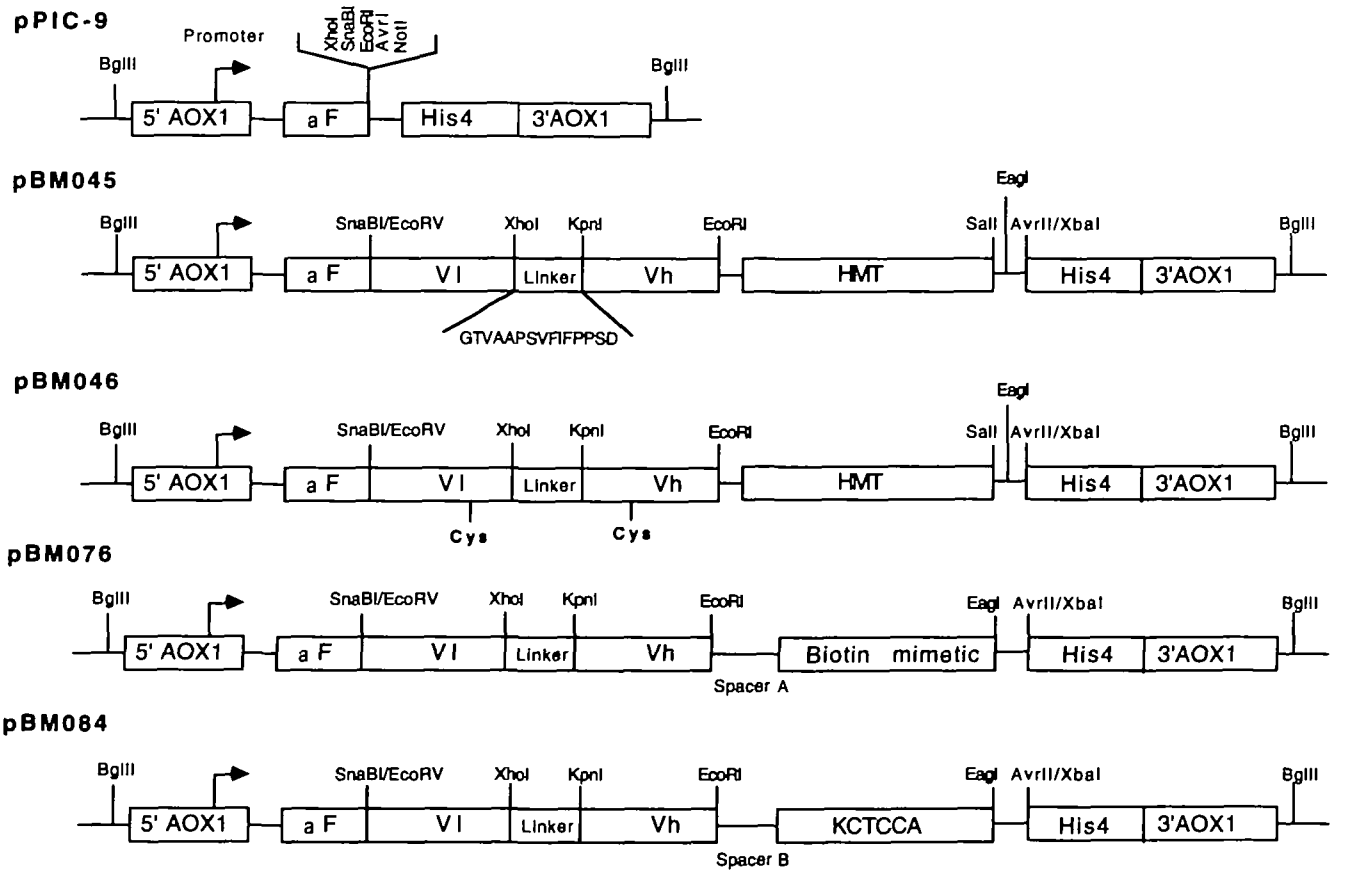

Fig. 2. **Plasmid construction of scFv-174 and dsFv-174 controlled by an inducible AOX1 gene promoter.** The plasmids derived from vector pPIC-9 contained an aF secretion signal. The amino acid sequence of the linker is given below the plasmid structure.

TABLE I. **Transformation of bi-functional scFv/dsFv constructs in *P. pastoris* cells.**

Plasmid name	Construct	Total number of His+ transformants	Total number of transformants picked	Number of His+, Mut− transformants	Protein expression	RIA[b]
pBM045	scFv-174-HMT	228	104	8 (7.7%)[a]	+	+
pBM046	dsFv-174-HMT	266	104	11 (10.6%)[a]	+	−
pBM076	scFv-174-KCTCCA	237	104	9 (8.6%)	+	+
pBM084	scFv-174-Biotin	212	104	6 (5.8%)	+	+

[a]Percentage of His+ Mut− (methanol utilization-deficient) transformants. [b]Radioimmunoassay.

screening for integration at the correct loci (*i.e.* that colonies grow on a −his/+glycerol plate but grow slowly on a −his/+methanol plate), all positive clones obtained (Table I) were isolated, cultured in induction medium, and then analyzed for protein expression. The biotin mimetic sequence isolated from phage display library on peptides (*15*) was used in this study as a target for marker-conjugated streptavidin (*16*). Figure 3 shows some representative results of detection of the scFv-174-biotin mimetic fusion protein using alkaline phosphase-conjugated streptavidin.

In order to demonstrate that the bi-functional scFv-174 and dsFv-174 proteins secreted by the recombinant *P. pastoris* cells have intrinsic binding activity, competitive radioimmunoassay (RIA) experiments were carried out, in which an anti-idiotype polyclonal antibody against MAb 174H.64 was coated on the solid phase, and ^{125}I labeled MAb 174H.64 was challenged with scFv-174 or dsFv-174 containing samples. The assay involving anti-idiotype antibodies provided a "mirror image" of the CA174 antigen, and it has been proved to be advantageous for obtaining highly reproducible results in RIA experiments. We found that scFv-174 samples isolated from pBM045-, pBM076-, and pBM084-transformed cells (scFv-174-HMT, scFv-174-biotin mimetic, and scFv-174-KCTCCA) showed positive binding activity, ranging from 66-82% inhibition on RIA, in comparison with 95% inhibitory activity of murine MAb 174H.64 (representative samples in Fig. 4), whereas none of the samples from pBM046-transformed cells (dsFv-174-HMT) showed any inhibitory activity. However, when the HMT fragments at the C-termini of scFv and dsFv were replaced by the c-myc sequence, the inhibitory activities of the two constructs on RIA were found to be similar (*13*). This indicates that the structural conformation of dsFv-174-HMT may be interrupted by the interactions between the cysteines we engineered in the Fv domains and the cysteines in the HMT region.

Previously, we introduced a disulfide bond into scFv-174 in an attempt to stabilize it (*13*). Our initial goal was to find the best connection between dsFv and a functional motif, to obtain a more stable bi-functional molecule. It has generally been believed that proteins secreted by eukaryotic cells, including *P. pastoris*, should have the most stable structure conformation. Therefore, they should, most likely, retain their biological functions. However, our finding of the loss of RIA binding activity by dsFv-174-HMT was a surprise, although this construct exhibited similar levels of protein expression and secretion to scFv-174-HMT. We noticed that the two proteins were indistinguishable when they were subjected to 10% SDS-PAGE (data not shown). Interestingly, both proteins (scFv-174-HMT *vs.* dsFv-174-HMT) exhibited dramatic differences

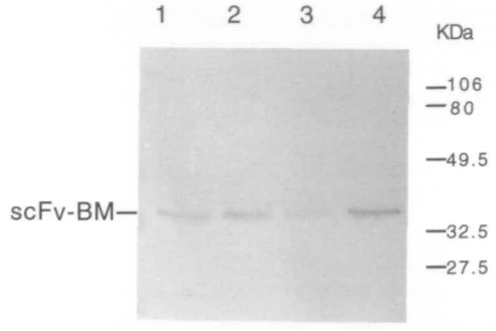

Fig. 3. Expression of the bi-functional protein of scFv-174-biotin mimetic (scFv-BM) in pBM076-transformed *P. pastoris* cells. Protein samples prepared from cell supernatants of representative clones (duplicates) were analyzed on 10% SDS-protein gels, followed by Western blotting using alkaline phosphatase-conjugated streptavidin at a final concentration of 2 μg/ml. The position of the protein is indicated.

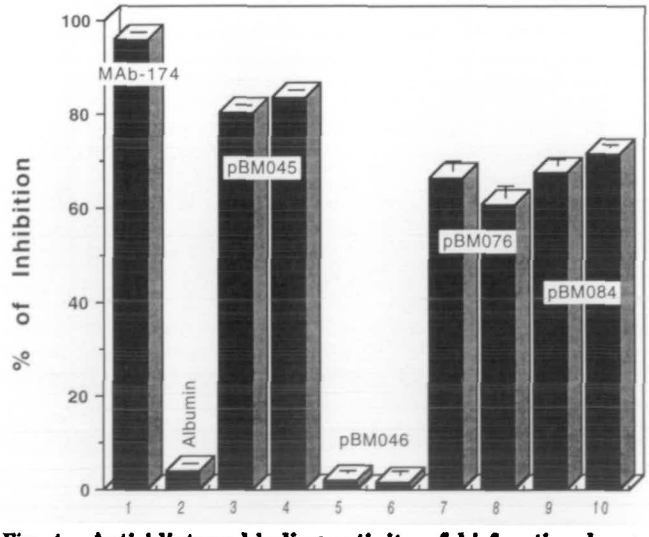

Fig. 4. Anti-idiotype binding activity of bi-functional proteins. A goat polyclonal anti-idiotype antibody was coated on the solid phase, and ^{125}I-labeled MAb 174H.64 was challenged with scFv-174-HMT, dsFv-174-HMT, scFv-174-biotin mimetic, and scFv-174-KCTCCA isolated from pBM045-, pBM046-, pBM076-, and pBM084-containing samples, respectively. Unlabeled MAb 174H.64 was used as a standard control, and human albumin produced in *P. pastoris* cells (Invitrogen) was used as a negative control.

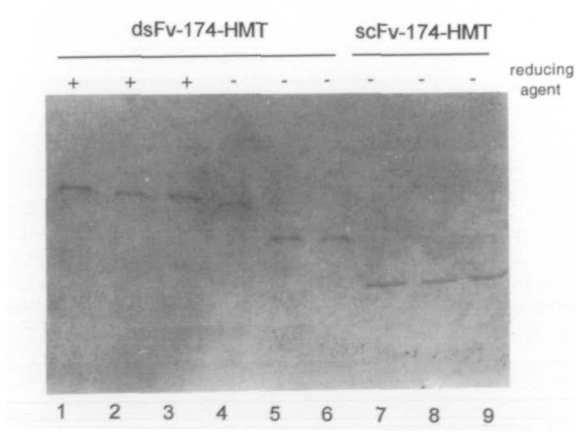

Fig. 5. Non-denaturing polyacrylamide gel electrophoresis of dsFv-174-HMT (lanes: 1-6) and scFv-174-HMT (lanes: 7-9). The scFv-174-HMT and dsFv-174-HMT containing samples (three independent samples) were first dialyzed three times against PBS and then concentrated on Centricon 10 columns (Amicon). β-Mercaptoethanol was added to some samples as a reducing agent (lanes: 1-3) before application to the gel. All samples were then directly applied to 10% non-denaturing polyacrylamide gels. After electrophoresis, the gels were stained with a Coomassie Blue solution.

under non-denaturing conditions, as shown in Fig. 5. The scFv-174-HMT and dsFv-174-HMT containing samples were first dialyzed three times against PBS, concentrated on Centricon 10 columns (Amicon), and then directly applied to 10% non-denaturing polyacrylamide gels. In contrast to three independent samples of scFv-174-HMT, which migrated to the same position on the gel, the dsFv-174-HMT containing samples showed heterogeneity of mobility on the gel (all three dsFv-HMTs had negative binding activity on RIA). However, when β-mercaptoethanol, a reducing agent, was added to them at the final concentration of 100 mM before application to the gel, three of them (dsFv-HMT) moved to even higher positions on the gel and they appeared to migrate to the same position (Fig. 5). This suggested that protein conformational changes might have occurred in these dsFv-HMT samples under non-denaturing conditions, and that the intramolecular disulfide linkages might have changed in the dsFv-HMT samples. Under reducing conditions, the disulfide bonds of dsFv-HMT were interrupted. Therefore, random conformations of the proteins could be expected. The difference in protein conformation between scFv-HMT and dsFv-HMT could explain why dsFv-HMT had lost the binding activity.

In addition to single-chain monospecific antibody, a bifunctional protein of a single-chain molecule may also be useful as a potential cancer imaging/therapeutic agent. The successful expression of a recombinant immunotoxin, a fusion protein of *Pseudomonas* exotoxin (*17-20*) or eosinophil-derived neurotoxin (*21*) and scFv or dsFv, has been shown. Recently, promising results were obtained with bi-specific scFv antibodies with novel biological properties and antitumor activity (*22*), although they had relatively high molecular sizes. Finally, the expression of native and fully active bi-functional scFv or dsFv in the *P. pastoris* system should provide an abundant source for future use in the diagnosis and treatment of human diseases.

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