

## Production of Functionalized Single-Chain Fv Antibody Fragments Binding to the ED-B Domain of the B-isoform of Fibronectin in *Pichia pastoris*

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The *Pichia pastoris* expression system was used to produce functionalized single-chain antibody fragments (scFv) directed against the ED-B domain of the B-fibronectin (B-Fn) isoform which was found to be present only in newly formed blood vessels during tumor angiogenesis. Therefore, scFv antibody fragments recognizing the ED-B domain are potential markers for angiogenesis. We constructed four functionalized scFv antibody fragments for direct labeling with radioactive molecules or toxins or for attachment to liposomes serving as carriers for cytotoxic or antiangiogenic compounds. The C-termini of the scFv antibody fragments contain 1–3 cysteine residues that are separated by a hydrophilic linker (GGSSGGSSGS) from the binding domain and are accessible for site-specific functionalization with thiol-reactive reagents. Plasmid expression, culture conditions, and purification were optimized in 1-L cultures. The scFv antibody fragments were purified by anion exchange chromatography. The yields were 5–20 mg/L culture medium. The large-scale production of one scFv antibody fragment in a 3.7-L fermenter gave a yield of 60 mg. The reactivity of the cyteines was demonstrated by labeling with the thiol-reactive fluorescent dye ABD-F. The four scFv antibody fragments bound specifically to ED-B-modified Sepharose and binding was further confirmed by immunofluorescence on cell cultures using ED-B-positive human Caco-2 tumor cells. Furthermore, we could demonstrate specific binding of scFv-modified liposomes to ED-B-positive tumor cells. Our results indicate that the *P. pastoris* expression system is

**useful for the large-scale production of cysteine-functionalized  $\alpha$ -ED-B scFv antibody fragments.**

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Single-chain Fv (scFV)<sup>2</sup> antibody fragments represent potential molecules for the targeted delivery of drugs, toxins or radionuclides and for coupling to liposomes for diagnostic and therapeutic applications. A scFv antibody fragment is an engineered antibody derivative that includes heavy- and light-chain variable regions joined by a peptide linker. ScFv antibody fragments are potentially more effective than unmodified IgG antibodies. The reduced size of 27–30 kDa permits them to penetrate tissues and solid tumors more readily (1). Carnemolla *et al.* (2) isolated and characterized a scFv antibody fragment binding to the human ED-B domain of the B-fibronectin isoform using phage display technology. Fibronectin (Fn) is an extracellular adhesion molecule that mediates interactions between cells and extracellular matrix components. Fn is a glycoprotein composed of two subunits joined by disulfide bonds. The protein is involved in different biological processes, such as establishment and maintenance of morphology, cell migration, hemostasis and thrombosis, wound healing, and oncogenic transformation (3). Fn polymorphism originates from alternative splicing patterns of the primary transcript of a single

<sup>2</sup> Abbreviations used: scFv, single-chain Fv; Fn, fibronectin; MM, methanol medium; YPD, yeast extract peptone dextrose medium; MD, minimal dextrose medium; TBP, tributylphosphine; ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DMSO, dimethyl sulfoxide; IPTG, isopropylthiogalactoside; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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gene in three regions (IICS, ED-A, ED-B) (3, 4). The B-Fn isoform with the ED-B oncofetal domain inserted by splicing is present in the stroma of fetal and neoplastic tissues and in adult and neoplastic blood vessels during angiogenesis but it is not detectable in mature vessels. (5, 6). Angiogenesis is defined as the formation of new capillaries from preexisting blood vessels. This process is essential for normal embryogenesis, reproduction, and growth, but it also occurs in the development of many diseases like rheumatoid arthritis, ischemic peripheral vascular disease, ischemic heart disease, diabetic retinopathy, and tumor growth (7, 8). Therefore, the ED-B isoform of Fn represents a promising marker for angiogenesis in growing solid tumors. The targeting properties of a radiolabeled human high-affinity antibody fragment (L19) directed against the ED-B domain of Fn was recently demonstrated by Tarli *et al.* (9). This affinity-matured scFv antibody fragment selectively accumulated in vessels of growing tumors of human and murine origin.

The methylotropic yeast *Pichia pastoris* has been shown to be suitable for the high-yield expression of various proteins by secretion into the culture supernatant or by intracellular localization (10, 11). Protein expression in *P. pastoris* is based on the use of the alcohol oxydase gene, AOX1. Transcription of the gene is regulated by the AOX1 promoter when the AOX1 coding sequence is replaced by a gene of interest. The activity of this promoter is strictly regulated by the concentration of the carbon source in the culture medium. Full transcriptional activity is achieved by addition of methanol. The protein yield can significantly be enhanced by manipulation of the factors that influence gene expression and product stability (12).

We cloned the sequence for the  $\alpha$ -ED-B scFv antibody fragments into the pPICZ $\alpha$ A yeast vector containing a signal peptide sequence needed for protein secretion, a Zeocin resistance gene, and a flag-tag sequence that facilitates detection of the protein. Cysteine engineering is a highly successful approach widely applicable to chemical modification (13, 14). Therefore, we constructed four  $\alpha$ -ED-B scFv antibody fragments that were different in the number of cysteines and spacer length between the cysteines and the sequence of the scFv antibody fragment. The scFv antibody fragments were functionalized for the direct attachment of drugs, toxins, radioisotopes or for the linkage to the surface of liposomes that can function as carriers of different molecules like anti-angiogenic and cytostatic drugs. Here we describe the construction of four  $\alpha$ -ED-B scFv antibody fragments containing additional cysteines at the C-terminus, their overexpression in *P. pastoris*, and their purification and characterization.

## MATERIALS

The pDN351 and pQE-12 plasmids were kind gifts of D. Neri, Department of Applied Biosciences, Swiss Federal Institute of Technology, Zurich. The pPICZ $\alpha$ A vector, the X-33 yeast strain, and the antibiotic Zeocin were obtained from Invitrogen BV (Leek, The Netherlands). All yeast culture media were from Difco (Detroit, MI). The oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). The Caco-2 (human colon adenocarcinoma) cell line was obtained from H. Wunderli-Allenspach (Dept. Applied Biosciences ETH, Zürich, Switzerland) and the Co-115 (human colon carcinoma) cell line was from B. Sordat (Swiss Institute for Cancer Research, ISREC, Lausanne, Switzerland). All buffer and salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

## METHODS

### *Construction of the Yeast Vectors*

The sequence of scFv- $\alpha$ -ED-B (CGS-1) was modified by PCR using pDN351 as a template (15). The forward primer (5'-CCGGAATTCGAGGTGCAGCTGGTGGAG 3') included an *Eco*RI site for cloning into the *P. pastoris* expression vector pPICZ $\alpha$ A. The reverse primers (pCM2: 5'-GCATCTAGATCATCATGAACACCCACATGACCCCTTGTCATCGTCGTCCTTGTAGTC-3'; pCM3: 5'-GCATCTAGATCAACATGAACACCCACATGACCCTGATGACCCCCCTGATGACCCCCCTTGTATCGTCCTTGTA-3'; pCM4: 5'-GCATCTAGATCATCAACAAACATGACCCTGATGACCCCTTGTCATCGTCGTCCTTGTAGTC-3'; pCM5: 5'-GCATCTAGATCATCAACATGACCCTGATGACCCCTGATGACCCCCCTTGTATCGTCGTCCTTGTAGTC-3') contained the sequence for the flag-tag, the hydrophilic spacer amino acids (GGSSGGSSGS) and the cysteines, a stop codon, and an *Xba* site for cloning into the *Xba* site of the *P. pastoris* expression vector. The reactions were performed in 50  $\mu$ L containing 120 ng of the DNA template, 25 pmol of each primer, 100  $\mu$ M dNTP (Boehringer Mannheim, Germany), and the Expand high-fidelity PCR system (1.75 U, Boehringer) was used for PCR. Twenty-five cycles were carried out in a DNA thermocycler (Perkin Elmer Certus), each consisting of 1 min at 94°C, 1 min at 55°C, and 75 s at 72°C. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Basel, Switzerland), digested with *Eco*RI and *Xba*, and purified by electrophoresis on agarose gels and with the QIAquick gel extraction kit (Qiagen). The four inserts were ligated into the pPICZ $\alpha$ A vector which had been digested with *Eco*RI and *Xba* and purified by phenol extraction. The ligations were performed in 30  $\mu$ L ligase buffer (Boehringer) containing 100 ng vector, 300 ng insert, and 1 U of T4 DNA ligase (Boehringer) for 12 h at 16°C.

### *Transformation of the pPICZ $\alpha$ A Vectors into E. coli*

Competent *E. coli* XL1blue bacteria were freshly prepared as described by Chang *et al.* (16). The four different plasmids (pCM2, pCM3, pCM4, pCM5, Fig. 1) were transformed into competent bacteria by incubation of the bacteria with the ligation mixture for 30 min on ice, 2 min at 42°C and again 2 min on ice. After addition of 1 mL low salt LB medium the bacteria were incubated for 1 h at 37°C. Transformed colonies were selected on low-salt LB plates containing 25  $\mu$ g/mL Zeocin. Six Zeocin-resistant clones of each construct were inoculated with 2 mL low-salt LB containing 25  $\mu$ g/mL Zeocin and grown overnight at 37°C. The DNA was isolated using the protocol of Holmes and Quigley (17). The plasmids containing an insert were identified by restriction digest analysis using *Bst*EII and *Sac*II. Clones containing the required insert were identified by DNA sequencing using the 5'AOX1 and the 3'AOX1 primers (Invitrogen). The four plasmids (pCM2, pCM3, pCM4, pCM5) containing the correct inserts were amplified using a plasmid maxiprep kit (Qiagen).

### *Transformation of the pPICZ $\alpha$ A Vectors into P. pastoris*

The four plasmids were linearized with *Sac*I transformed into the *P. pastoris* strain X-33 by electroporation. The X-33 yeast cells were grown overnight at 30°C in 5 mL yeast extract peptone dextrose medium (YPD; 1% yeast extract, 2% peptone, 2% dextrose). Fresh YPD medium (500 mL) were inoculated with the overnight cultures and incubated until an  $A_{600nm}$  of 1 was reached. The cells were sequentially centrifuged (5 min, 1500g) and the pellets resuspended in 500 mL sterile ice-cold water, 250 mL sterile ice-cold water, 2 mL sterile ice-cold 1 M sorbitol, and 1 mL sterile ice-cold 1 M sorbitol, respectively. The competent cells were electroporated by pulse discharge (1500 V, 25  $\mu$ F, 200  $\Omega$ ; Genepulser, BioRad, Switzerland) in the presence of 7  $\mu$ g of each linearized plasmid. After adding 1 mL sorbitol (1 M) the cultures were incubated for 1 h at 30°C without shaking. The electroporation mixtures were plated on YPDS (YPD with 1 M sorbitol) plates containing 100  $\mu$ g/mL Zeocin. After 72 h incubation at 30°C, 20 clones of each construct were picked and spotted on minimal methanol medium (MM) agar plates (1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, 0.5% methanol), on minimal dextrose medium (MD) agar plates (1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, 2% dextrose) and on YPDS containing different amounts of Zeocin (2000, 1000, 500, and 100  $\mu$ g/mL). After growing for 3 days at 30°C transformants which were growing on MD medium and on medium containing 2000  $\mu$ g Zeocin but only very slowly on MM medium were retained for further characterization.

### *Screening for High-Expression Clones*

Five positive clones of each construct were used to inoculate 10 mL BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 100 mM potassium phosphate (pH 6), 1.34% yeast nitrogen base with ammonium sulfate and without amino acids) in 50 mL tubes overnight at 30°C. These cultures were used to inoculate 100 mL BMGY medium in 250-mL shaker flasks for 48 h. The cells were centrifuged (10 min, 2000g) and the pellets resuspended in 100 mL BMMY medium (BMGY containing 0.5% methanol instead of glycerol) in 250 mL shaker flasks for 72 h. To induce expression, the cultures were supplemented every 24 h with methanol to a final concentration of 0.5% (v/v). Culture supernatants were sampled at different time points to monitor the scFv antibody fragments production by Western blot analysis. The clones with the highest production clones were stored in 25% glycerol at -70°C.

### *Large-Scale Protein Production*

One single clone of each of the X-33 constructs was picked and grown overnight in 10 mL BMGY. This overnight culture was used to inoculate 500 mL BMGY in a 1-L shaker flask for 48 h at 30°C and 250 rpm until the culture reached an  $A_{600nm}$  of 15. To induce the scFv antibody fragment production the cells were centrifuged (10 min, 2800g) and the pellets resuspended in 500 mL BMM (BMMY without yeast extract) containing 0.001% Structol (Schill + Seilacher, Hamburg, Germany) as an antifoam agent. The cells were shaken in a 1-L baffled flask for 72 h at 30°C and 250 rpm. For scFv production, cultures were supplemented every 24 h with methanol to a final concentration of 0.5% (v/v). Culture supernatants were sampled to monitor the scFv yield.

### *Production of scFv-CM3 in a Fermenter*

One single clone of pCM3/X-33 was picked and grown overnight in 5 mL BMGY. This overnight culture was used to inoculate 250 mL BMGY in a 1-L shaker flask for 48 h at 30°C and 250 rpm until the culture reached an  $A_{600nm}$  of 15. This inoculum was transferred into a 3.7-L fermenter (Bioengineering, Wald, Switzerland) containing 1.3 L synthetic medium (580 mL 10 $\times$  basal salt (containing in 1 L: 52 mL phosphoric acid, 1.8 g CaSO<sub>4</sub>  $\times$  2H<sub>2</sub>O, 28.6 g K<sub>2</sub>SO<sub>4</sub>, 6.5 g KOH, 7 g MgSO<sub>4</sub>  $\times$  7H<sub>2</sub>O) plus 7.8 mL trace salt solution (containing in 1 L: 6 g CuSO<sub>4</sub>  $\times$  5H<sub>2</sub>O, 0.08 g NaI, 3 g MgSO<sub>4</sub>  $\times$  H<sub>2</sub>O, 0.2 g MoNa<sub>2</sub>O<sub>4</sub>  $\times$  2H<sub>2</sub>O, 0.02 g H<sub>3</sub>BO<sub>3</sub>, 0.5 g CoCl<sub>2</sub>, 20 g ZnCl<sub>2</sub>, 65 g FeSO<sub>4</sub>  $\times$  7H<sub>2</sub>O) plus 70 g glycerol, 0.2 g biotin, and 5 mL sulfuric acid (25%). After 12 h a glycerol feed (50% (v/w)) containing 12 mL/L trace salts was started at 10 mL/h for the

duration of 12 h. The temperature was kept at 30°C and dissolved oxygen maintained above air saturation. The pH was adjusted to 5.5 with 20% ammonium hydroxide. The protein production was induced after 30 h inoculation and an  $A_{600\text{nm}}$  of 100 by addition of methanol. The methanol concentration was kept constant at 0.5% (v/v) final concentration and the production stopped after 24 h.

#### *Purification of scFv Antibody Fragments*

The yeast cells were removed by centrifugation (10 min, 2800g) and the cell free medium further clarified through a 0.2- $\mu\text{m}$  filter. The proteins were either precipitated or the supernatant directly loaded on an anion exchange column. The proteins were precipitated by addition of ammonium sulfate at 80% saturation for 1 h on ice under slow stirring. Precipitated proteins were pelleted by centrifugation (20 min, 14,000g at 4°C) and resuspended in PB (67 mM phosphate buffer, pH 7.4). Ammonium sulfate was removed by dialysis against PB overnight at 4°C and the remaining protein solution loaded on a DEAE-Sephacel anion exchange column (2  $\times$  10 cm, Pharmacia Biotech) equilibrated with PB. After washing the column with PB the protein was eluted with a step gradient of 0–250 mM NaCl in PB with steps of 50 mM NaCl at 4°C. Fractions of 5 mL were collected and analyzed by SDS–PAGE. The amount of protein was determined using the BioRad Protein Assay. The yields were 5 to 20 mg/L protein depending on the particular constructs.

#### *Gel Electrophoresis and Western Blot Analysis*

Gel analysis of the proteins was performed as described by Laemmli (18), using 16% acrylamide gels followed by staining with Coomassie brilliant blue or immunoblotting. For Western blot analysis the proteins were transferred from the gels onto an Immobilon-P transfer membrane (Millipore) using a semidry system (1 h, 0.8 mA/cm<sup>2</sup>) in transfer buffer (48 mM Tris base, 39 mM glycine, 0.0375% SDS, 20% methanol). The membranes were soaked for 1 h in TBST (10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween 30) containing 5% milk powder followed by a 1 h incubation in TBST containing 1% milk powder (TBSTM) containing an anti-flag M2 antibody (1:2000 dilution) (Integra Biosciences, Wallisellen, Switzerland). Membranes were washed three times for 10 min with TBST and incubated for 1 h with an alkaline phosphatase-labeled goat anti-mouse IgG (1:7000 dilution) (Promega, Zürich, Switzerland) in TBSTM. After three washings with TBST the proteins were detected with a ProtoBlot II AP system (Promega).

#### *Cysteine Labeling of the scFv Antibody Fragments*

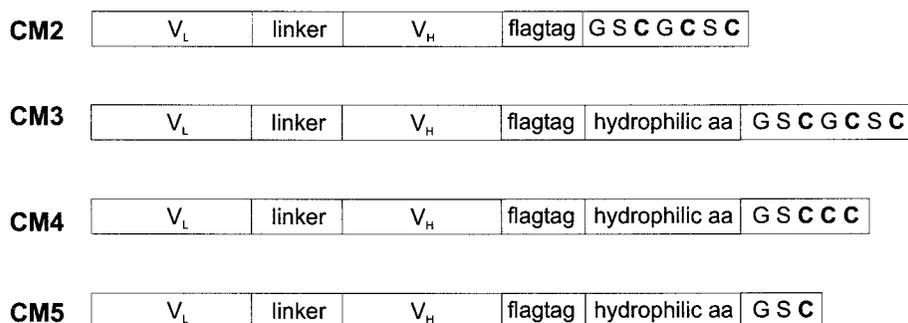
Stock solutions of 40 mM tributylphosphine (TBP, Fluka, Buchs, Switzerland) and 40 mM 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F, Molecular Probes, Eugene, OR) in DMSO were prepared immediately before use. The labeling of the protein (0.5 mg/mL) was performed in PB containing 2 mM TBF and 4 mM ABD-F at 30°C for 15 min (19). The labeled proteins were loaded onto 16% SDS–polyacrylamide gels and after electrophoresis the gels were photographed using a transilluminator UV (312 nm) light source and subsequently stained with Coomassie staining.

#### *Production and Purification of the ED-B Peptide*

The production and purification of the ED-B peptide was performed as described by Carnemolla (2). Briefly, *E. coli* (TG1) transformed with the pQE-12 vector containing the sequence for ED-B were grown at 37°C in 2  $\times$  TY medium (1.6% tryptone, 1% yeast extract, 85 mM NaCl) containing 100  $\mu\text{g}/\text{mL}$  ampicillin, 25  $\mu\text{g}/\text{mL}$  kanamycin, and 1% glucose. When the cell suspension reached an  $A_{600\text{nm}}$  of 0.8, isopropylthiogalactoside (IPTG, Appligene, Basel, Switzerland) was added to a final concentration of 1 mM and growth continued overnight at 30°C. After centrifugation (30 min, 4000g), the pellet was resuspended in 60 mL sonication buffer (PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>) containing 20 mM imidazole and 250 mM NaCl) and sonicated three times for 1 min on ice. The soluble protein was separated from insoluble compounds by centrifugation (20 min, 17,000g). The supernatant with the protein was incubated with 4 mL Ni-NTA (Qiagen) resin equilibrated in sonication buffer for 30 min on ice, filled into a column (1.5  $\times$  10 cm), and washed with sonication buffer. The ED-B peptide was eluted with PBS containing 200 mM imidazole and 250 mM NaCl. The fractions were analyzed on a nonreducing 23% SDS–PAGE gel and the protein was stored at –20°.

#### *Specific Binding of scFv Antibody Fragments to ED-B*

ED-B was linked to CNBr-activated Sepharose 4B (Pharmacia Biotech) by incubation of 4 g gel with 30 mL ED-B (0.6 mg/mL) in coupling buffer (0.1 M NaHCO<sub>3</sub>, pH 8.6, 0.5 M NaCl) for 1 h. The reaction was periodically controlled by measurement of the  $A_{280\text{nm}}$  of the supernatant and analysis on a nonreducing 23% SDS–polyacrylamide gel. After incubation with the ED-B protein, the resin was washed in succession with 5 gel volumes of 0.1 M acetate (pH 4.0), 0.5 M NaCl, followed by 0.1 M Tris–HCl (pH 8) and 0.5 M NaCl. The scFv antibody fragments (0.1 mg) in their dimeric form were loaded onto the ED-B Sepharose column. The column was washed with PBS, and the scFv antibody fragments eluted with 100 mM triethylamine in PBS.



**FIG. 1.** Scheme of the  $\alpha$ -ED-B scFv antibody fragment constructs containing different C-termini. Hydrophilic spacer amino acids (GGSSGGSSGS). V<sub>L</sub>, variable light chain; V<sub>H</sub>, variable heavy chain. The boxes are not drawn to scale.

### *In Vitro* Binding to ED-B-Positive Tumor Cells

Caco-2 cells (ED-B positive) were maintained in DMEM (Gibco, Basel, Switzerland) supplemented with heat-inactivated 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100  $\mu$ g/mL streptavidin. Co115 cells (ED-B negative) were maintained in the same medium without nonessential amino acids and pyruvate. Cover slips (20 mm diameter) were placed into 12 well plates and were coated with 100  $\mu$ L rat tail collagen I (10 mg/mL) which was isolated from rat tails as described by Elsdane (20) by incubation for 30 min at 37°C. Caco-2 and Co115 cells ( $3 \times 10^5$  cells/well) were plated and cultured for 72 h in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Washed cells were incubated with scFv-CM3 (5  $\mu$ g/well) in 100  $\mu$ l PBS containing 3% bovine serum albumin (BSA) for 30 min at 4°C. After two washings with PBS the cells were incubated with the anti-flag M2 antibody (1  $\mu$ g/well, Integra Biosciences) followed by a FITC-labeled rabbit anti-mouse IgG (1  $\mu$ g/well, Southern Biotechnology, Birmingham, AL). After another washing step the cover slips were removed, treated with 10% glycine and placed on a microscope slide and analyzed on a fluorescence microscope (Leica DLMB). As negative controls the cells were incubated with the M2 antibody followed by the FITC labeled rabbit anti-mouse IgG or the FITC labeled rabbit anti-mouse IgG alone.

## RESULTS

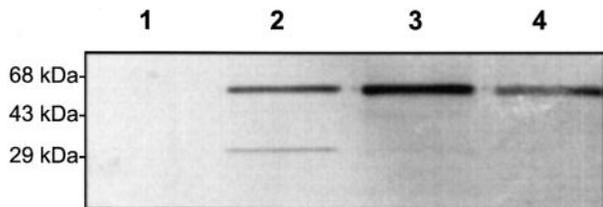
### *Construction and Transformation of the Vectors*

The sequence of the  $\alpha$ -ED-B scFv antibody fragment (CGS-1) was modified by PCR at the 5'-end by introduction of an *Eco*RI site and at the 3'-end of the sequences for the different C-termini, a flag-tag, and a *Xba* site, respectively (Fig. 1). The 0.7-kb inserts were ligated into the multiple cloning site region downstream of the *P. pastoris* AOX1 promoter and the  $\alpha$ -factor signal sequence of the pPICZ $\alpha$ A vector using the *Eco*RI/*Xba* restriction sites. The resulting plasmids

(pCM2, pCM3, pCM4, pCM5) were transformed into the *Escherichia coli* strain XL1blue. The pPICZ $\alpha$ A plasmid contains the Zeocin resistance gene for selection in *E. coli* and *P. pastoris*. Plasmids carrying the insert were selected on low salt LB plates containing Zeocin. Three hundred transformants were recovered. The 3'- and 5'-nucleotide sequences of one positive clone of each construct were confirmed by DNA sequencing using 3'- and 5'-AOX1 sequencing primers. The pPICZ $\alpha$ A vector has one *Sac*I restriction site in the AOX1 locus. Vectors linearized with *Sac*I will integrate into the host's 5'-AOX1 region. Thus, the linearized plasmids were introduced into the yeast X-33 strain by electroporation. The recombinants were selected on YPDS plates containing Zeocin. Several hundred colonies were obtained. Five clones of each construct were selected for high Zeocin (2 mg/mL) resistance on YPDS plates and for slow growth on minimal methanol medium, indicating that homologous recombination with the AOX-1 gene has occurred.

### *ScFv Antibody Fragments Expression, Production, and Purification*

Expression of the scFv antibody fragments was assessed in a small volume culture experiment. The protein production of totally 20 clones of each construct was tested by analysis of the yeast culture supernatants by Western blotting. All tested clones secreted the scFv antibody fragments into the supernatant but in different amounts. The clones of each construct with the highest yields were chosen for upscaled protein production. A small overnight culture was used to inoculate 1 L BMGY medium. The cells were grown for 48 h and when they had reached an  $A_{600nm}$  of 15 the medium was changed to BMM for induction of protein production. The large-scale protein production was done in medium without yeast extract (BMM) for easier purification. To prevent protein degradation the concentration of methanol was kept at 0.5%. The use of baffled flasks gave a higher yield compared to conventional bottles. The pH value was critical and optimized

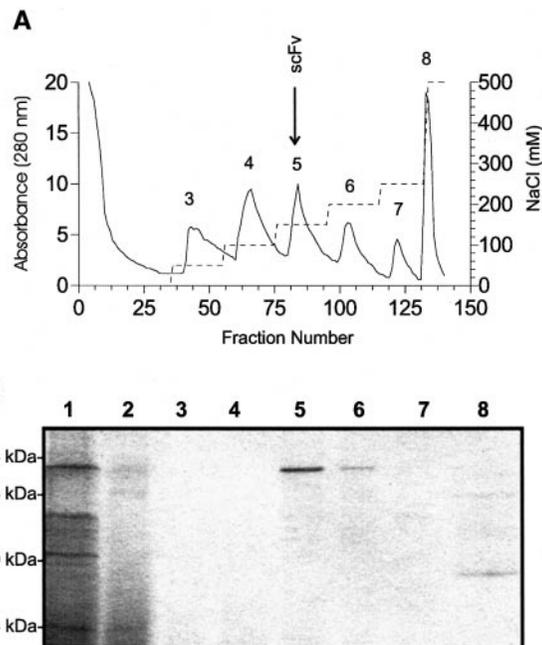


**FIG. 2.** Western blot of the supernatant during scFv-CM3 production in *P. pastoris*. Samples were taken at different time points after methanol induction. Lane 1, 0 h; lane 2, 24 h; lane 3, 48 h; lane 4, 72 h. (Comparable results were obtained with the other scFv antibody fragments.) Lower band, monomer; upper band, dimer. Non-reducing gel.

for the production of the scFv antibody fragments. At pH values of  $>6$  proteases were active, whereas at pH values below 5 protein degradation by hydrolysis was observed. Based on these observations the production was started at pH 5.5 and, if necessary, the pH adjusted frequently. The duration of the protein production was also critical. As shown in Fig. 2 the protein (scFv-CM3) was detected after 24 h in the supernatant in its monomeric as well as in its dimeric form. The highest yield of protein was obtained after 48 h. No protein was found in the cell pellets. The isolation of the scFv antibody fragments by ammonium sulfate precipitation was detrimental for the proteins. Consequently, purification on an anion exchange column with elution by a stepwise NaCl gradient was chosen (Fig. 3A). The eluted fractions were analyzed by a SDS-polyacrylamide gel (Fig. 3B) and confirmed by Western blots (data not shown). The majority of the purified protein eluted at 150 mM NaCl and the yield was 5–20 mg/L culture medium. The four scFv antibody fragments were concentrated to 0.5 mg/mL and stored at  $-20^{\circ}\text{C}$ . For further scaling-up the scFv-CM3 was produced in a 3.7-L fermenter. Based on the results of the production in the baffled flasks the production in the fermenter was stopped after 24 h. The yield of the production was 60 mg.

#### Characterization of the scFv Antibody Fragments

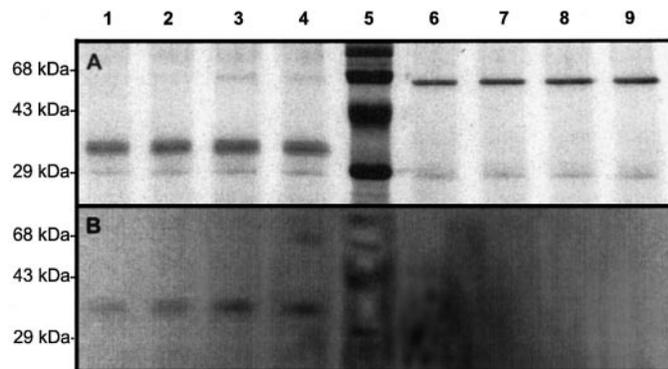
In contrast to the unmodified scFv antibody fragments produced in *E. coli* the functionalized scFv proteins produced in yeast formed dimers. The scFv antibody fragments were reduced to monomers after treatment with TBP (Fig. 4A, lanes 1–4). This indicated that the C-terminal cysteines were accessible for chemical modification. To confirm this observation the cysteine groups were modified with the fluorescent thiol reactive dye ABD-F (19). As shown in Fig. 4B only the reduced four scFv antibody fragments reacted with ABD-F and were detectable under UV-light as faint fluorescent bands.



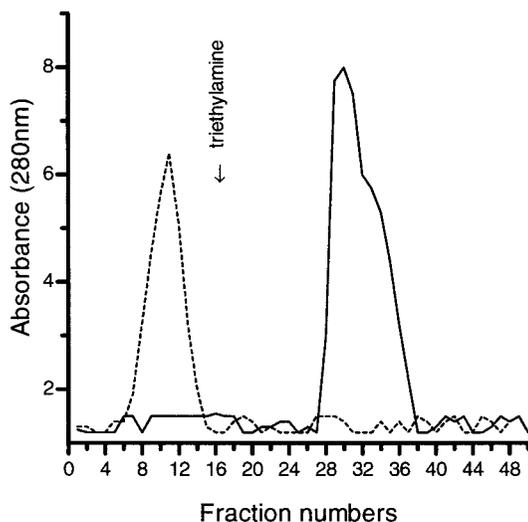
**FIG. 3.** Purification of the scFv-CM3. (A) Elution profile of the scFv antibody fragment purification on a DEAE-Sephacel column. The scFv antibody fragment was eluted with a step gradient of NaCl in 67 mM phosphate buffer, pH 7.4 (dotted line), and protein was monitored at 280 nm (full line). (B) Coomassie-stained SDS-polyacrylamide gel analysis of the scFv antibody fragment after column purification. Lane 1, supernatant before purification; lane 2, flowthrough fractions; lane 3, fractions eluted with 50 mM NaCl; lane 4, fractions eluted with 100 mM NaCl; lane 5, fractions eluted with 150 mM NaCl; lane 6, fractions eluted with 200 mM NaCl; lane 7, fractions eluted with 250 mM NaCl; lane 8, fractions eluted with 500 mM NaCl.

#### Binding of the scFv Antibody Fragments to ED-B

The binding of the four scFv antibody fragments were tested on an ED-B modified Sepharose column



**FIG. 4.** Labeling of the thiol groups of the four scFv antibody fragments with ABD-F. (A) Coomassie-stained SDS-polyacrylamide gel. (B) Fluorescence of the ABD-labeled scFv antibody fragments photographed using UV light. Reducing conditions: lane 1, scFv-CM2; lane 2, scFv-CM3; lane 3, scFv-CM4; lane 4, scFv-CM5; lane 5, marker. Nonreducing conditions: lane 6, scFv-CM2; lane 7, scFv-CM3; lane 8, scFv-CM4; lane 9, scFv-CM5.



**FIG. 5.** Elution profile of the  $\alpha$ -ED-B scFv-CM3 (solid line) and an irrelevant scFv antibody fragment (dotted line) on an ED-B-modified Sepharose column. The scFv antibody fragment was eluted with 100 mM triethylamine.

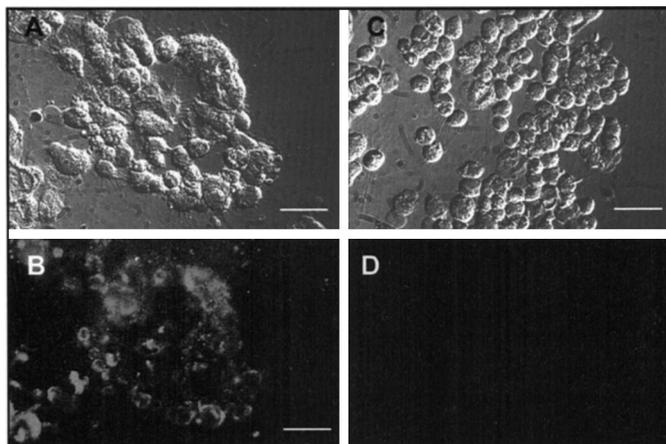
and in cell cultures on ED-B positive tumor cells. Figure 5 shows the binding properties of the dimeric form of scFv-CM3 and of an irrelevant, scFv antibody to immobilized ED-B. The irrelevant scFv antibody fragment eluted in the void volume of the column, whereas the scFv-CM3 antibody was released from ED-B after addition of triethylamine. The other scFv antibody fragment dimers (scFv-CM2, scFv-CM4, and scFv-CM5) had comparable binding properties to ED-B Sepharose. Caco-2 tumor cells express ED-B fibronectin (21) that allowed us to demonstrate the specific binding of the scFv antibody fragment dimers *in vitro*. As shown in Fig. 6 binding of the scFv-CM3 antibody fragment to ED-B positive Caco-2 cells but not to the ED-B negative Col15 control cells could be demonstrated by immunofluorescence.

## DISCUSSION

We constructed four functionalized  $\alpha$ -ED-B scFv antibody fragments with or without hydrophilic spacers and different numbers of cysteines located at their C-termini with the purpose to prepare functionalized scFv antibody fragments differing in length of inserted spacer and number and position of introduced cysteines at the C-terminal end of the proteins (Fig. 1). The free thiol groups of the scFv monomers allow the attachment of various molecules and/or liposomes that can serve as carriers for therapeutic drugs, toxins, radioisotopes, or fluorescent markers. Production and purification of the  $\alpha$ -ED-B scFv antibody fragments in the *P. pastoris* yeast expression system was established and the specific binding of the scFv to the ED-B domain of fibronectin demonstrated.

The ease of the expression and purification of functional scFv antibody fragments in *P. pastoris* confirm the potential of this system for producing quantities of protein needed for therapeutic purposes. Recently, other scFv antibody fragments have been expressed in *P. pastoris* at large but variable yields (11, 22, 23). Thus, protein expression levels depend on copy numbers and the sites of integration as well as on fermentation parameters. The production of large amounts of proteins of high purity and stability becomes an important issue for the development of therapeutic recombinant proteins. Proteins like endostatin (24, 25), IL-17 (26, 27), and insulin (28) were produced in the *P. pastoris* system.

C-terminal cysteine thiols offer a large variability for site-specific modifications of the scFv antibody fragment molecules having the advantage of not interfering with antigen binding domains. The modification of proteins with bispecific coupling molecules (e.g., the thiol-reactive reagents SATA or SPDP) that interact with amino groups on the protein could potentially reduce the binding activity of the antibody fragments. Furthermore, thioether linkages formed between cysteines and maleimide reagents have a better *in vivo* stability than disulfide linkages that is a prerequisite for organ-specific targeting. The utility of liposomes as a drug delivery system is well established (29). Targeting of liposomes via surface-coupled antibodies represents an effective method to modify their biodistribution and/or pharmacokinetic parameters (30, 31). The  $\alpha$ -ED-B scFv antibody fragments will be used for the preparation of immunoliposomes that serve as carriers



**FIG. 6.** Specific binding of the  $\alpha$ -ED-B scFv-CM3 antibody fragment to cells cultured for 48 h on collagen I-coated coverslips. (A, C) Phase contrast images of the sections shown in B and D. (A, B) Caco-2 cells (ED-B fibronectin positive). (C, D) Col15 cells (ED-B fibronectin negative). Specific binding was detected by immunofluorescence with the anti-flag-tag antibody M2 followed by a FITC-labeled anti-mouse antibody (B, D). Original magnification, 400 $\times$ , bar = 100  $\mu$ m.

for therapeutic or diagnostic molecules (32, 33). In preliminary experiments, we linked the  $\alpha$ -ED-B scFv fragments CM2, CM3, CM4, and CM5 via thioether coupling to poly(ethylene glycol)-maleinimide-modified small unilamellar liposomes. The coupling efficiency of the fragments to small unilamellar liposomes of 50 nm mean diameter gave an average of 30 scFv molecules linked to one liposome for the CM2, CM4, and CM5 constructs, whereas with construct CM3 an average of 70 fragment molecules could be attached to one liposome. In a recent study Midulla *et al.* (34) analyzed location and source of ED-B Fn in human tumor- and endothelial-derived cell lines, as well as in human breast and colorectal tumor tissue samples. ED-B Fn was found to be expressed in 6 of 10 colorectal and in 19 of 20 breast tumor tissue samples of mixed histotypes and in normal and transformed endothelial cell lines. Furthermore, ED-B Fn was produced and secreted by human epidermoid carcinoma KB cells in a mouse xenograft model. These data suggest that tumor cells can act as a source of ED-B Fn, representing a potential target for specific tumor targeting. Using the scFv-CM3-modified liposomes, we were able to demonstrate specific binding to the ED-B domains of Fn on Caco-2 cells *in vitro* as well as enhanced antitumor activity of these liposomes *in vivo* in a mouse tumor model (35).

Thus, by combination of the attachment of tumor and endothelial cell specific molecules to drug carrying liposomes an effective system for simultaneous and targeted treatment of tumors and their vasculature will be available.

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