Expression of a bispecific dsFv–dsFv′ antibody fragment in Escherichia coli

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A bispecific disulfide-stabilized Fv antibody fragment (dsFv–dsFv′) consisting of two different disulfide-stabilized Fv antibody fragments connected by flexible linker peptides was produced by secretion of three polypeptide chains into the periplasm of Escherichia coli. The dsFv–dsFv′ molecules were enriched by immobilized metal affinity chromatography and further purified by anion-exchange chromatography. The recombinant antibody constructs retained the two parental antigen binding specificities and were able to cross-link the two different antigens. The described dsFv–dsFv′ design might be of particular value for therapeutic in vivo applications since improved stability is expected to be combined with minimal immunogenicity.

Keywords: antibody engineering/bispecific antibodies/bivalent antibodies/dsFv/recombinant antibodies

Abbreviations: Fv, antibody fragment consisting of the variable region of light and heavy chain; V_H, variable region of heavy chain; V_L, variable region of light chain; dsFv, disulfide-stabilized Fv antibody fragment; phOx, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one

Introduction

The variable region (Fv) of an antibody, comprised of the variable heavy (V_H) and the variable light chain (V_L) domain, is the smallest antibody fragment containing a complete antigen binding site. Many native Fv antibody fragments, however, have insufficient stability because the V_H and V_L domain can rapidly dissociate, which is usually accompanied by aggregation (Glockshuber et al., 1994). Therefore, different strategies have been developed to stabilize the association of these domains. The most widespread solution is to connect the V_H and V_L domain by a short peptide linker between the carboxy-terminus of one domain and the amino-terminus of the other to form a single-chain Fv antibody fragment (scFv) (Bird et al., 1988; Huston et al., 1988; Whitlow and Fipula, 1991). In comparison with the much larger complete IgG, scFv antibody fragments have lower retention times in non-target tissues, a more rapid blood clearance and better tumor penetration in vivo (Milenic et al., 1991; Yokota et al., 1992; Adams et al., 1993; Colcher et al., 1999). They are therefore useful for some diagnostic and therapeutic applications, e.g. for the targeted delivery of radionuclides, drugs or toxins to a tumor site. The improved stability of scFv antibody molecules when compared with Fv fragments is mainly achieved by preventing free dissociation of the V_H and V_L domain and thus increasing their local concentrations during the association reaction rather than by a structural effect. On the other hand, the linker mediates the tendency of scFv fragments to aggregate at higher concentrations. This obstacle was overcome by the strategy of directly linking the scFv and V_L domain at their contact surface by a covalent connection instead of utilizing a linker peptide. To achieve this, cysteine residues have been genetically engineered into complementary framework positions of the V_H and the V_L domain distant from the CDRs to form disulfide bond-stabilized Fv antibody fragments (dsFvs) (Brinkmann et al., 1993; Reiter et al., 1995). These molecules are very stable and usually retain full antigen binding activity. Sometimes, they have even better antigen binding affinities than their scFv homologs (Reiter et al., 1994a–c; Webber et al., 1995).

In recent decades, recombinant antibody technologies were developed which allow the addition of new functions to the antigen binding domains of immunoglobulin derivatives. The production of various Fab, scFv or dsFv fusion proteins with heterologous partners has been reported (Breitling and Dübel, 2000). Further, homologous fusions, i.e. bispecific antibodies, allowed the cross-linking of two different antigens, e.g. on two different cells. These molecules are particularly useful to redirect T-cells to tumor cells that are not originally recognized by the cellular immune system (Hombach et al., 1993; Arndt et al., 1999). The application of hybrid hybridoma (quadroma) technology initially developed to produce bispecific antibodies was hampered by problems of cell line stability, yield and purification of the desired antibody since the co-expression of two antibodies in one cell leads to the production of 10 different heavy and light chain combinations. Therefore, various recombinant approaches have been employed to generate bivalent and bispecific antibodies in heterologous expression systems (Breitling and Dübel, 2000; Dübel and Kontermann, 2000). Particularly promising is the diabody format (Holliger et al., 1993). Bispecific diabodies are heterodimers consisting of two scFv antibody fragments in which the V_L and the V_H domains of the two Fv antibody fragments are present on different polypeptide chains. The oligopeptide linkers between these domains are too short to allow pairings between V_H and V_L domains at the same chain (Perisic et al., 1994; Poljak, 1994). Diabodies have been employed in immunoassays (Kontermann et al., 1997a; Watkins et al., 1999) or to recruit C1q, resulting in efficient lysis of antigen-coated sheep erythrocytes (Kontermann et al., 1997b). Bispecific diabodies with one arm directed against the target antigen and the other against the serum Ig were able to recruit complement, induce mononuclear phagocyte respiratory burst and phagocytosis and promote synergistic cytotoxicity towards colon carcinoma cells in conjunction with CD8+ T-cells (Holliger et al., 1997). Other diabodies have been used successfully to target directly cytotoxic T-cells to tumor markers, lymphoma cells or cytokine...
dition. Further, diabodies are usually not very flexible since they have two linker peptides between the Fv fragments which have to be very short to prevent monomer formation. Some Cys(100)V L(215) was generated by introducing the Cys

It was therefore suggested to combine two dsFv antibody fragments by a single, flexible linker to obtain bispecific disulfide-stabilized antibody molecules (Breitling and Dubel, 1997a). The covalent connection of two dsFv molecules should improve the stability of the antibody construct by providing covalent coupling of the antibody polypeptides. Further, it should improve flexibility by using a single linker peptide which may be longer than in diabodies since it is not necessary to keep it short to ensure dimerization. Finally, these two improvements can potentially reduce the immunogenicity of the protein in a therapeutic approach.

Improved stability and tumor localization were reported for diabodies with disulfide cross-linked V H and V L domains of an anti-CEA antibody (FitzGerald et al., 1997). These constructs, however, still required two short linker peptides. Recently, Bera et al. (1998) reported the production of a bivalent Fv immunotoxin based on the assembly of two polypeptides. In this construct, the V H and the V L domains of the anti-erbB2 antibody e23 were covalently linked to each other by a disulfide bond. The two V H domains of the dsFvs are connected by a single flexible polypeptide linker. They further fused this bivalent, but not bispecific (dsFv)2 antibody molecule to PE38, a truncated form of Pseudomonas exotoxin, to generate a recombinant immunotoxin.

In this paper, we describe for the first time the construction and functional expression of a bispecific dsFv–dsFv’ construct, which was generated by connecting two disulfide-stabilized Fv antibody fragments of different antigen specificity by a single 10 amino acid residue linker.

Materials and methods

Vector construction

All standard cloning procedures were carried out as described by Sambrook et al. (1989). The construction of the vectors pDOPE51–215b were mutated which led to the vector pDOPE51–215(GS). To obtain pDOPE511–215, the dsFv(215) coding region was cleaved out of pDOPE51–215b with NcoI (New England Biolabs) and NorI and ligated to the vector fragment of pDOPE51–215(GS) cut with the same restriction enzymes. Finally, the vector pDOPE511–Cys(105)V H(phOx)/Cys(100)V L(215) was generated by introducing the Cys(105)V H(phOx) coding region from pDOPE51–phOx into the plasmid pDOPE51–215 using the restriction enzymes NcoI and HindIII. The vector pOPE101–Cys(44)V H(215)–Yol–Cys(43)V L(phOx) was obtained by introducing the Cys(44)V H(215)–Yol–Cys(43)V L(phOx) coding region from pOPE51–Cys(44)V H(215)–Yol–Cys(43)V L(phOx) into the plasmid pOPE101–215(Yol) using the restriction enzymes EcoRI and BglII (New England Biolabs). To obtain pDOPE111–Cys(105)V H(phOx)/Cys(100)V L(215) the vector pOPE111–215 was digested with KpnI and StuI (New England Biolabs). The resulting fragment was ligated to the vector pDOPE511–Cys(105)V H(phOx)/Cys(100)V L(215) cleaved with the same restriction enzymes. The vectors pOPE101–Cys(44)V H(215)–n-Cys(43)V L(phOx) (n = 10, 8, 5 or 3) were obtained by exchanging the linker coding region of pOPE101–Cys(44)V H(215)–Yol–Cys(43)V L(phOx) with the respective insert sequences coding for a 10, eight, five or three amino acid residue linker. New linker coding regions were generated by the synthesis of the overlapping oligonucleotide forward primers 5’–TCA GGT GGA GGC GGT TCA GAT or 5’–TCA GGT GGA GGC GGT TCA GAT, 5’–TCA GGT GGA GGC GGT TCA GAT or 5’–TCA GGT GGA GGC GGT TCA GAT and the reverse primers 5’–ATC GCC TCC ACC TGG ACC TCC ACC, 5’–ATC GCC TGG ACC TCC ACC, respectively. The corres-

Unwanted restriction sites for NcoI in the kanamycin resistant gene and MluI in the lac Ig gene of the resulting vector pDOPE51–215b were mutated which led to the vector pDOPE51–215(GS). To obtain pDOPE511–215, the dsFv(215) coding region was cleaved out of pDOPE51–215b with NcoI (New England Biolabs) and NorI and ligated to the vector fragment of pDOPE51–215(GS) cut with the same restriction enzymes. Finally, the vector pDOPE511–Cys(105)V H(phOx)/Cys(100)V L(215) was generated by introducing the Cys(105)V H(phOx) coding region from pDOPE51–phOx into the plasmid pDOPE51–215 using the restriction enzymes EcoRI and BglII (New England Biolabs). To obtain pDOPE111–Cys(105)V H(phOx)/Cys(100)V L(215) the vector pOPE111–215 was digested with KpnI and StuI (New England Biolabs). The resulting fragment was ligated to the vector pDOPE511–Cys(105)V H(phOx)/Cys(100)V L(215) cleaved with the same restriction enzymes. The vectors pOPE101–Cys(44)V H(215)–n-Cys(43)V L(phOx) (n = 10, 8, 5 or 3) were obtained by exchanging the linker coding region of pOPE101–Cys(44)V H(215)–Yol–Cys(43)V L(phOx) with the respective insert sequences coding for a 10, eight, five or three amino acid residue linker. New linker coding regions were generated by the synthesis of the overlapping oligonucleotide forward primers 5’–TCA GGT GGA GGC GGT TCA GAT or 5’–TCA GGT GGA GGC GGT TCA GAT, 5’–TCA GGT GGA GGC GGT TCA GAT or 5’–TCA GGT GGA GGC GGT TCA GAT and the reverse primers 5’–ATC GCC TCC ACC TGG ACC TCC ACC, 5’–ATC GCC TGG ACC TCC ACC, respectively. The corres-

All vectors and ligation products were electrotransferred into Escherichia coli XL1-Blue competent cells (Stratagene, La Jolla, CA) and clones with open reading frames were identified by colony lifts (Breitling and Dubel, 1997b) on Protran BA 85 nitrocellulose (0.45 mm, Schleicher und Schuell, Dassel, Germany) after induction with 100 µM IPTG and immunostaining with mouse monoclonal antibody Myc1–9E10 (Calbiochem, Cambridge, UK). The correct insertion of each gene fragment in productive clones was confirmed by restriction analysis and partial sequencing. The production of antibody
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Fig. 1. pOPE and pDOPE vector constructs employed in this study. Mutations of framework positions to cysteine are indicated. Abbreviations: Amp r, gene providing ampicillin resistance; His, sequence encoding for six histidine residues; IR, intergenic region of phage f1; Kan r, gene providing kanamycin resistance; lac Iq, lac repressor; myc, epitope decapetide of the monoclonal antibody Myc1–9E10; pelB, signal peptide sequence of bacterial pectate lyase, mediating secretion into the periplasmic space; RBS, ribosome binding site; Ser A, N-terminal epitope recognized by serum ‘A’; Stop, stop codon; V H and V L, heavy and light chain variable region coding sequences; Yol, epitope of the monoclonal antibody Yol 1/34. The elements of the insert regions are not drawn to scale.

constructs after induction with 20 µM IPTG (when using pOPE vectors) or 100 µM (when using pDOPE vectors) for 3 h was confirmed by electrophoresis of total cell lysates on 12% polyacrylamide SDS gels followed by immunoblotting and by ELISA.

SDS–PAGE and immunoblot analysis
SDS–PAGE was carried out according to Laemmli (1970) on 12% polyacrylamide gels stained with Coomassie Brilliant Blue R250 (Serva, Heidelberg, Germany). Immunoblots were performed according to Towbin et al. (1980). Unspecific antibody binding was blocked with 2% (w/v) skimmed milk powder (Oxoid, Basingstoke, UK) in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4, pH 7.3) containing 0.05% (v/v) Tween 20 (MPBST). The mouse monoclonal antibody Myc1–9E10 (Calbiochem) recognizing the c-myc-tag, the rat monoclonal antibody Yol1/34 (Harlan Sera-lab, Sussex, UK) recognizing the Yol-tag or a rabbit serum ‘A’ recognizing exclusively the processed N-terminus of the V H domain (Dübel et al., 1992) were used as primary antibodies for immunodetection (1/1000 in MPBST). Subsequently, HRP-conjugated antibodies to mouse, rat or rabbit immunoglobulins (Dianova, Hamburg, Germany) were applied (1/2000 in MPBST). TMB Stabilized Substrate for HRP (Promega, Madison, WI) was used for visualizing bound enzymatic activity.

Functional expression of antibody fragments into the periplasm of E.coli
Overnight cultures of E.coli cells transformed with combinations of pDOPE and pOPE vectors (Figure 1) were diluted 1:20 into LB-medium containing 100 µg/ml of ampicillin (Biomol, Hamburg, Germany), 50 µg/ml of kanamycin (Serva) and 100 mM glucose and grown at 37°C to an OD600 nm of 0.6. The promoters were induced by the addition of 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG). After an additional 3 h of shaking at room temperature, the bacteria were harvested by centrifugation at 5000 g for 10 min. The total cell lysate was analyzed by SDS–PAGE followed by immunoblotting using the mouse monoclonal antibody Myc1–9E10 for immunodetection.

Extraction of soluble Fv fragments from the periplasm
For the preparation of periplasmic extracts, the harvested bacteria were resuspended in 1/10 of the original culture volume of spheroblast solution [50 mM Tris–HCl (pH 8.0), 20% (w/v) sucrose and 1 mM EDTA], left for 20 min on ice with occasional shaking and harvested by centrifugation at 4°C and 6200 g for 10 min. The supernatant representing the periplasmic enriched fraction was cleared by centrifugation at 4°C and 30 000 g for 30 min, dialyzed against PBS and used for functional studies in ELISA. The bacterial pellet was resuspended in 1/10 of the original culture volume of 5 mM MgSO4 and left for 20 min on ice with occasional shaking. After centrifugation at 4°C and 30 000 g for 30 min, the supernatant representing the osmotic shock fraction was also dialyzed against PBS.

Enrichment of soluble Fv fragments from periplasmic extracts
The fusion protein was further enriched by immobilized metal affinity chromatography (IMAC) utilizing the carboxy-terminal His-tag of the V L domains. The IMAC column, containing a 2 ml bed volume per liter of original bacterial culture of chelating Sepharose (‘fast flow’ loaded with NiCl2; Pharmacia, Uppsala, Sweden), was equilibrated with PBS containing...
To determine the antigen binding activity of the different was isolated from a phage antibody library and improved by ELISA.

µWiesbaden, Germany) were coated overnight at 4°C. After washing the column with 5 ml of 30 mM Tris-HCl (pH 7.8), the antibody fragments were further purified by anion-exchange chromatography using Mono Q HR 5/5 (Amersham Pharmacia, Uppsala, Sweden). Protein-containing fractions obtained from IMAC were pooled, dialyzed against 30 mM Tris-HCl (pH 7.8) and applied to the column equilibrated with the same buffer. After washing the column with 5 ml of 30 mM Tris–HCl (pH 7.8), the antibody fragments were eluted from the column using a biphase NaCl gradient in 30 mM Tris–HCl (pH 7.8) increasing to 250 mM in 20 ml, then from 250 mM to 1 M NaCl in 2 ml. For final elution, 5 ml of 1 M NaCl in 30 mM Tris–HCl (pH 7.8) were applied. The fractions were analyzed by SDS–PAGE followed by immunoblotting using mouse monoclonal antibody Myc1–9E10 (Calbiochem). The Fv antibody fragments were then dialyzed twice against PBS and 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, the reaction short sequences coding for a flexible polypeptide linker. To study the effect of the linker length on the folding properties of the antibody constructs, we mutated two different pairs of the variable domains, we mutated two different pairs of the amino acid residues (Figure 1). The gene encoding the heavy and the light chain of the dsFv(215) antibody fragment and the light chain of the dsFv(215) antibody were genetically connected by introducing the mutations Lys(105)Cys into the VH domain and Ala(43)Cys into the VL domain. Cloning, expression and characterization of the two separate dsFv molecules have been described previously (Schmiedl et al., 1992). To avoid mispairings of the variable domains, we mutated two different pairs of conserved, complementary framework positions distant from the CDRs for disulfide stabilization of the two different Fv fragments. The disulfide bond-stabilized Fv(215) antibody fragment, dsFv(215), was generated by the mutations of the amino acid Ser(44)Cys of the VH and Ala(100)Cys of the VL domain. The dsFv(phox) antibody fragment was obtained by introducing the mutations Lys(105)Cys into the VH domain and Ala(43)Cys into the VL domain. Cloning, expression and characterization of the two separate dsFv molecules have been described previously (Schmiedl et al., 2000).

To determine the bispecificity of the antibody constructs, 96-well Immuno Plates (MaxiSorp F96; Nunc) were coated overnight at 4°C with 0.1 µg of phoxylated BSA in 100 µl of 0.1 M NaHCO₃ buffer (pH 8.0) per well. Unspecific antibody binding was blocked with 400 µl of MPBS for 6 h at room temperature. Volumes of 100 µl of fractions containing the antibody fragments were added per well. HRP-labeled streptavidin [Amersham Pharmacia, Braunschweig, Germany; 8:1000 in PBS, 0.05% (v/v) Tween 20], which was pre-incubated with 0.1 nM of a biotinylated 215 epitope peptide (biotin–LPHFIKDDYGPESRGFVENSYLAGLTPSE), was used for detection. Another biotinylated peptide (biotin–KESRAKKFQRQHMDEGEFF) served as a control. Biotinylated peptides were supplied by the central peptide synthesis facility of the Zentrum für Molekulare Biologie, Heidelberg, Germany. TMB was used for the detection of bound enzymatic activity.

**Results**

**Vector construction**

We designed a compatible set of two E.coli vectors to allow the production of three polypeptide chains necessary to form a bispecific disulphide-stabilized Fv–Fv’ antibody molecule (Figure 1). In the resulting molecule, the heavy and the light chain of each of the two different Fv fragments are expected to be connected to each other by an interdomain disulfide bond (see Figure 7, bottom).

The dsFv antibody fragments used in the construct were derived from the mouse monoclonal antibody clone 3 (= mAb215) (Krämer et al., 1980; Kontermann et al., 1995) binding to the largest subunit of RNA polymerase II of Drosophila melanogaster and from an scFv antibody fragment specific for the hapten 2-phenylloxazolin-5-one (phox), which was isolated from a phage antibody library and improved by chain shuffling (Marks et al., 1992). To avoid mispairings of the variable domains, we mutated two different pairs of conserved, complementary framework positions distant from the CDRs for disulfide stabilization of the two different Fv fragments. The disulfide bond-stabilized Fv(215) antibody fragment, dsFv(215), was generated by the mutations of the amino acid Ser(44)Cys of the VH and Ala(100)Cys of the VL domain. The dsFv(phox) antibody fragment was obtained by introducing the mutations Lys(105)Cys into the VH domain and Ala(43)Cys into the VL domain. Cloning, expression and characterization of the two separate dsFv molecules have been described previously (Schmiedl et al., 2000).

To generate the bispecific antibody fragment, the heavy chain of the dsFv(215) antibody fragment and the light chain of the dsFv(phox) antibody were genetically connected by short sequences coding for a flexible polypeptide linker. To assess the effect of the linker length on the folding properties of the complete molecule, we generated a set of vectors encoding bispecific dsFv(215)–dsFv(phox) antibody constructs with linkers consisting of three, five, eight or 10 amino acid residues (Figure 1). The gene encoding the Cys(44)VL(215) domain was connected to the gene for the Cys(43)VL(phox) domain by respective oligonucleotides and cloned into the pDPOE vector, a derivative of pDM1.1 (Lanzer, 1988). The genes encoding for the Cys(105)VL(phox) domain and the Cys(100)VL(215) domain were cloned into the expression vector pOPE111 (Schmiedl et al., 2000). Each of the two genes was separately cloned in-frame between a pelB-leader sequence (Lei et al., 1987) for the secretion of the antibody fragments into the periplasmic space and a small coding region.
for C-terminal tags facilitating detection and purification. In case of the Cys(105)\(\text{VH}(\text{phOx})\) domain, the tag contains the linear epitope ‘EEGEFSEAR’ recognized by the mouse monoclonal antibody mAb Yol1/34 (Kilmartin et al., 1982; Breitling and Little, 1986). At the C-terminus of both \(\text{VH}\) domains, a short peptide tag of the proto-oncogene product \(c\)-myc was located containing the linear epitope ‘EELI-SEEDL’ recognized by the mouse monoclonal antibody mAb Myc1–9E10 (Evan et al., 1985) followed by six histidine residues. The resulting vector \(\text{pOPE111–VH}(\text{phOx})/\text{VL}(215)\) and the set of four different variants of \(\text{pOPE101–VH}(215)–n\)-\(\text{VL}(\text{phOx})\) \((n = 10, 8, 5\) or 3) encoding different linkers between their variable heavy and light chain regions are shown in Figure 1. The two backbones encode for compatible origins of replication and different resistance markers. A combination of a \(\text{pOPE}\) and \(\text{pDOPE}\) vector can thus be propagated in the same \(E.\text{coli}\) cell after co-transfection.

To confirm the function of the individual vectors first, all plasmids were separately electrotransferred into \(E.\text{coli}\) XL1-Blue competent cells. The production of the antibody components after induction of their promoters with 100 \(\mu\)M IPTG (when using the vector \(\text{pDOPE101}\) or 20 \(\mu\)M IPTG (when using \(\text{pOPE111}\)) for 3 h was confirmed by electrophoresis of total cell lysates on 12% polyacrylamide SDS gels followed by immunoblotting using the mouse monoclonal antibody mAb Myc1–9E10 for immunodetection (data not shown).

**Expression of the recombinant bispecific dsFv(215)–dsFv(phOx) antibody construct**

\(E.\text{coli}\) XL1-Blue competent cells were co-transfected with one of the plasmid variants of \(\text{pDOPE101–VH}(215)–n\)-\(\text{VL}(\text{phOx})\) containing different linker lengths \((n = 10, 8, 5\) or 3) and \(\text{pOPE111–VH}(\text{phOx})/\text{VL}(215)\) to express the three polypeptides required to form the bispecific disulfide bond-stabilized antibody construct within the same cell. The production of antibody fragments after induction with 100 \(\mu\)M IPTG for 3 h was confirmed by electrophoresis of total cell lysates of induced and non-induced culture samples on 12% polyacrylamide SDS gels (Figure 2A) followed by immunoblotting using the mouse monoclonal antibody Myc1–9E10 for immunodetection (Figure 2B). After induction, the expected bands corresponding to the processed translation product Cys(44)\(\text{VH}(215)–n\)-Cys(43)\(\text{VL}(\text{phOx})\) (calculated molecular mass 27.4 kDa for \(n = 10\)) as well as the processed translation product Cys(100)\(\text{VH}(215)\) (calculated molecular mass 14.8 kDa for \(n = 10\)) were detected. The two bands appear to be produced in similar amounts as concluded from their almost identical intensities. The production of the Cys(105)\(\text{VH}(\text{phOx})\) domain (calculated molecular mass 15.3 kDa) was confirmed by immunoblotting using the rat monoclonal antibody Yol1/34 (data not shown). To confirm the observations in respect of the processing, immunoblots were further analyzed with rabbit serum ‘A’, which recognizes the sequence QVQLQ located at the N-terminus of the \(\text{VH}\) (215) domain (Dübel et al., 1992). Serum ‘A’ does not react with QVQLQ when presented as an internal epitope and can therefore be used to confirm the proteolytic removal of the signal sequence from the antibody fragment. The \(\text{VH}\) domain of the \(\text{phOx}\) antibody fragment does not contain the QVQLQ epitope and could not be detected in this approach. However, the expression and the correct processing of a substantial fraction of the \(\text{VH}\) domain of the dsFv 215 molecule could be confirmed (Figure 2C).

To confirm the production of soluble antibody fragments in the correct cell compartment, reduced periplasmic extracts of induced and non-induced bacteria were analyzed on 12% polyacrylamide gels followed by immunoblotting (Figure 2). A significant amount of proteins could be detected utilizing mAb Myc1–9E10 (Figure 2B) and serum ‘A’ (Figure 2C), thus demonstrating the expression of the antibody components as soluble proteins in the periplasmic space.

**Functional bispecific dsFv(215)–dsFv(phOx) molecules can be produced in \(E.\text{coli}\)**

To check for a correct folding and assembly of all three polypeptide chains, separate binding studies by ELISA on both antigens (215 epitope β-galactosidase fusion protein and phoxylated BSA) were performed. First, periplasmic extracts of induced and non-induced cultures were prepared and subjected to antigen ELISA. Bound antibody fragments were detected by their \(c\)-myc peptide tag. All dsFv fragments retained the antigen binding specificity of the original antibodies. (Figure 3A). The antibody construct containing the 10 amino acid residue linker showed the strongest binding activity to both of the two antigens.

To determine whether both antigen binding sites were present within a single bispecific molecule, phoxylated BSA was bound to ELISA wells and incubated with periplasmic extracts. Bound antibody fragments were detected with a small biotinylated peptide containing the 215-epitope, that was pre-incubated with HRP-labeled streptavidin. All of the four constructs provided bispecific binding activity. Again, the construct containing the 10 amino acid residue linker showed the strongest antigen cross-linking activity (Figure 3B).

After proof of principle for the production of bispecific dsFv–dsFv’ antibody molecules, the antibody construct containing the 10 amino acid residue linker was selected for further studies. Different IPTG concentrations were employed to determine the optimum IPTG concentration for the production. The highest amount of functional protein was obtained after induction with 100 \(\mu\)M IPTG as shown by ELISA (Figure 4). Higher IPTG concentrations led to larger amounts of total protein, but an increasing portion of the expressed antibody fragments still carried the leader sequence and formed aggregates (data not shown). This observation is in good accordance with results obtained for the production of scFv antibody fragments from a related vector (Dübel et al., 1992).

**Purification of dsFv(215)–dsFv(phOx) molecules**

Following these observations, the disulfide-stabilized, bispecific antibody construct containing the 10 amino acid residue linker was purified from the periplasm after induction with optimized IPTG concentrations. As a first purification step, the antibody fragment was enriched from periplasmic extracts by IMAC (Lilius et al., 1991). The fractions contained a significant amount of \(E.\text{coli}\) proteins. Therefore, they were further subjected to ion-exchange (Mono Q) chromatography (Figures 5 and 6).

Seven peaks (A–G) were observed (Figure 5A) and the corresponding protein fractions were analyzed by electrophoresis on 12% polyacrylamide gels followed by immunoblotting under reducing (Figure 6A) and non-reducing (Figure 6B) conditions. The three components of the bispecific antibody molecule with a calculated molecular mass of 57.5 kDa, namely the \(\text{VH}(215)–\text{VL}(\text{phOx})\) linker construct, \(\text{VH}(215)\) domain and the \(\text{VL}(\text{phOx})\) domain were detected by immunoblotting under reducing conditions (Figure 6A). Under non-reducing conditions (Figure 6B), fractions 11 and 12 (peak D) contained a
Fig. 2. SDS-PAGE and immunoblot analysis of *E. coli* co-transfected with pOPE111–VH(phOx)/VL(215) and with one of the vector derivatives of pDOPE101–VH(215)–n-VL(phOx) (n = 10, 8, 5 or 3). Analysis of total bacterial cell lysates (left) and periplasmic extracts (right) after 3 h of shaking with (+) and without (−) induction with IPTG. (A) Total protein stained by Coomassie Blue; (B) immunoblot stained with mAb Myc1–9E10; (C) immunoblot stained with rabbit serum ‘A’.

protein with the apparent molecular weight of 57 kDa, which consisted of the VH(215)–VL(phOx)-linker-construct, the VL(215) domain and of the VH(phOx) domain as confirmed by immunoblotting using mAb Myc1–9E10 and mAb Yol1/34. We conclude that fractions 11 and 12 (peak D) contained the majority of the bispecific dsFv(215)–dsFv(phOx) antibody construct. Fraction 11 contained also a significant amount of a 29 kDa protein, which is not the VH(215)–VL(phOx)-linker-construct of pDOPE as determined under reducing conditions. This protein is potentially a dimer of the VL(215) domain and, since it is also present in fraction 10, we concluded that it did not contribute to the bispecificity demonstrated by ELISA (Figure 5C). The other peaks contained mainly 215 light chain domains (peaks A, B and C) or contaminating *E. coli* proteins (peaks E, F and G). To determine the antigen binding activities of the different fractions, 100 µl samples were analyzed by single antigen as well as by cross-linking ELISA as described. In support of the immunoblot analysis, fractions 11 and 12 were shown to contain the strongest antigen binding to the 215-antigen and to phoxylated BSA (Figure 5B), as well as in the ELISA setup determining the antigen cross-linking (Figure 5C). The yield of the bispecific antibody construct was determined to be 0.1 µg/l of *E. coli* culture after purification.

**Discussion**

We constructed, produced and purified a new type of recombinant bispecific antibody in which both VH–VL heterodimers were stabilized by an interdomain disulfide bond. The VH domain of one dsFv molecule and the VL domain of the other dsFv fragment were cross-linked by a single, flexible polypeptide linker, thus forming a bispecific, disulfide-stabilized Fv–Fv’ antibody (dsFv–dsFv’, Figure 7). Binding studies demonstrated that the molecule retained the binding specificity of the two parental antibodies. The molecule was further able to cross-link the two antigens.

The results demonstrate for the first time that three different antibody domains can be covalently assembled to form a functional molecule in the periplasm of *E. coli*. However, careful balancing of the production rates of all three polypeptide chains is essential. Before we came to the final design of the vectors as described in Figure 1, we had generated a set of vector derivatives with the respective antibody fragment coding inserts exchanged between the pOPE and pDOPE vectors. In these cases, however, the Cys(44)VH(215)–n-Cys(43)VL(phOx) polypeptide chains were expressed to a higher amount in comparison with the Cys(100)VL(215) chains. We obtained similar 215-epitope and phOx-binding activities with crude periplasmic extracts but only a low antigen cross-linking activity, indicating the prevalence of products with only one correctly formed dsFv fragment.

Several approaches have been used previously to generate recombinant bivalent or multivalent Fv antibody molecules. The protein designs include disulfide-linked antibody fragments, mini-antibodies and diabodies (Cumber et al., 1992; Gruber et al., 1994; Hayden et al., 1994; De-Jonge et al., 1995; Holliger et al., 1996). To produce bivalent Fab (Rodrigues et al., 1993) or scFv molecules (Adams et al., 1993, 1995; McCartney et al., 1995), an unpaired cysteine located at the C-terminus of the antibody fragment has frequently been employed. Oxidation led to the formation of an interdomain disulfide bond.
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Fig. 3. Antigen binding activity of periplasmic fractions of E.coli co-transfected with pOPE111–VH(phOx)/VL(215) and with one of the vector derivatives of pDOPE101–VH(215)–n-VL(phOx) (n = 10, 8, 5 or 3) after 3 h induction with IPTG. (A) 215-epitope binding activity was determined using the 215-epitope β-galactosidase fusion protein as antigen. An analogous construct of β-galactosidase fused to a peptide comparable in size but not containing the 215-epitope served as a specificity control. Phoxylated BSA was used to determine phOx binding activity. BSA was used as a negative control. Bound antibody constructs were detected using mAb Myc1–9E10 and anti-mouse immunoglobulin-HRP conjugates. (B) Antigen cross-linking activity was determined in a sandwich ELISA using phoxylated BSA as antigen. Bound antibody constructs were detected with HRP-labeled streptavidin that was pre-incubated with the biotinylated 215-epitope peptide. A peptide comparable in size but not containing the 215-epitope served as a specificity control.

Fig. 4. Influence of the IPTG concentration on antigen binding activity of crude periplasmic extracts before and after 3 h induction with IPTG. (A) ELISA determining the binding to both antigens separately, as described in Figure 3. (B) Antigen cross-linking ELISA as described in Figure 3.

bond between the two fragments, thus generating bivalent antibodies with enhanced affinity. However, the stability under physiological conditions has not yet been assessed. Further, the unpaired cysteine led to problems with E.coli production (Schmiedl et al., 2000). To generate bivalent or multivalent Fv fusion proteins, a variety of self-associating secondary structures such as helix bundles, coiled coils or hybridizing oligonucleotides were further investigated (Pack and Plückthun, 1992; Plückthun, 1992; Plückthun and Pack, 1997; Zahida et al., 1999). Tetrapodal mini-antibodies were designed by attaching the modified leucine zipper sequences at the carboxy-terminal end of scFv fragments via a hinge region or by introducing a helical dimerization domain between two scFv fragments (Müller et al., 1998a). Again, the stability of these constructs under physiological conditions has not been reported and all constructs contained substantial heterologous domains that may give rise to an immune reaction against these domains which may interfere with therapeutic applications. The latter is to a much lesser extend expected from the minibody format conceived first by Hu et al. (1996). The fusion of an scFv molecule directed against carcinoembryonic antigen (CEA) to the C_H3 domain of an IgG1 led to the formation of bivalent molecules with excellent tumor-targeting properties such as rapid and high tumor uptake combined with rapid blood clearance. A similar approach to produce bispecific mini-antibodies was reported by Müller et al. (1998b), who fused the C_H1 domain of an IgG1 to an scFv fragment and the C_L domain of a kappa light chain to another scFv fragment. The domain assembly led to a functional bivalent and bispecific mini-antibody.

Another strategy for the production of bispecific antibody molecules without fusion of the Fv fragment to heterologous domains is the production of scFv–scFv’ antibodies (‘tandem antibodies’) (Mallender et al., 1994a,b). Two scFv molecules of different specificity were covalently cross-linked via a flexible polypeptide linker. These molecules were able to bind two adjacent epitopes on the same antigen (Neri et al., 1995) or cross-linking antigens on two different cells (Kurucz et al., 1995). Of similar size but consisting of two separate polypeptide chains is the diabody design with two antigen binding sites (Holliger et al., 1993) which can be bivalent or bispecific. Bispecific diabodies are dimers of two ‘cross-over’ scFv fragments in which the V_H and V_L domains of the two parent antibodies are present on different polypeptide chains. A potential disadvantage of this design is that the polypeptide linkers between these domains have to be shorter than in scFv fragments to prevent pairings between domains on the same chain (Kortt et al., 1997). High-level diabody secretion of functional diabodies from E.coli is possible, as demonstrated
by the recovery of a humanized bispecific diabody in yields approaching 1 g/l (Zhu et al., 1996) and has been improved by domain interface engineering (Zhu et al., 1997). Humanized or human diabodies pose little risk of immunogenicity since the only non-natural sequences are the short linkers between variable domains (Carter and Merchant, 1997); even these can be humanized by recruiting residues from the C\texttt{H}\texttt{C} (Holliger et al., 1996). The major disadvantage of both the tandem and the diabody design is that the domain interfaces of the two Fv fragments are not covalently cross-linked and thus prove the dissociation-related aggregation typical for all scFv derivatives. This problem has recently been addressed by the production of diabodies in which one Fv domain is disulfide stabilized (ds diabodies). These constructs confirmed the predictions obtained from monovalent dsFv fragments that a higher stability and improved tumor localization can be expected after disulfide stabilization (FitzGerald et al., 1997).

In general, owing to the necessity to have two very short linkers between their variable domains, diabodies are rigid and not very flexible, which may lower the chances of obtaining optimum binding to both antigens to be cross-linked in an in vivo situation. The new design described in this paper addresses this potential disadvantage, as well as the stability and immunogenicity issue, in trying to combine proven features of previous designs (Figure 7). Therefore, the dsFv–dsFv' is

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**Fig. 5.** Mono Q chromatography. (A) Elution profile. The antibody fragments were eluted from the column using a biphasic NaCl gradient in 30 mM Tris–HCl (pH 7.8) increasing to 250 mM in 20 ml, then from 250 mM to 1 M in 2 ml. For final elution, 5 ml of 1 M NaCl in 30 mM Tris–HCl (pH 7.8) were applied. The proteins were collected in 800 µl fractions using a flow-rate of 0.5 ml/min. (B) ELISA determining the binding to both antigens separately, as described in Figure 3. (C) Antigen cross-linking ELISA as described in Figure 3.

**Fig. 6.** SDS-PAGE and immunoblot analysis of reduced (A) and non-reduced (B) fractions obtained after Mono Q chromatography. Total protein was stained with Commassie Brilliant Blue. MAb Myc1–9E10 was used for immunodetection.

**Fig. 7.** Developments in the design of small bivalent and bispecific antibody constructs based on homologous fusions.
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