# The first constant domain ($C_H1$ and $C_L$) of an antibody used as heterodimerization domain for bispecific miniantibodies

Kristian M. Müller[a], Katja M. Arndt[a], Wolfgang Strittmatter[b], Andreas Plückthun[a,*]

[a] *Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland*
[b] *Merck KGaA, D-64271 Darmstadt, Germany*

Received 24 December 1997

**Abstract Bispecific miniantibodies were constructed by genetically fusing the $C_H1$ domain of an IgG1 to the C-terminus of a single-chain Fv fragment (scFv-425), specific for the EGF receptor, and fusing the $C_L$ domain of a kappa light chain to the C-terminus of a scFv specific for CD2 (scFv-M1). An efficient dicistronic gene arrangement for functional expression in *Escherichia coli* was constructed. Immunoblots demonstrated correct domain assembly and the formation of the natural $C_H1$-$C_L$ disulfide bridge. Gel filtration confirmed the correct size, sandwich ELISAs demonstrated bispecific functionality, and SPR biosensor measurements determined binding to EGF-R in comparison to bivalent constructs. Bispecific anti-EGF-R/anti-CD2 miniantibodies are candidates for the immunotherapy of cancer.**



*Key words:* Bispecific antibody; scFv fragment; Tumor targeting; CD2; EGF receptor

## 1. Introduction

We present here a novel approach to generate bispecific miniantibodies which functionally assemble in *Escherichia coli*. They are based on single-chain Fv fragments (scFv), in which the variable domain of the heavy chain ($V_H$) is connected by a glycine-rich linker to the variable domain of the light chain ($V_L$) [1–3]. Two different scFv fragments are brought together by fusing them, via a hinge region, to the first constant domain of the heavy chain ($C_H1$) or the constant domain of the κ light chain ($C_L$) domain of an antibody. The $C_L$ and $C_H1$ domains form a covalently linked heterodimer carrying two different scFv specificities, named $C_HC_L$ miniantibody. This work extends earlier work on multivalent, but homospecific miniantibodies [4–6], in which small oligomerization motifs such as amphipathic helices have been used to create molecules with high avidity.

Bispecific antibodies have been considered as potential drugs for tumor therapy, mainly for the induction of an antibody dependent cell mediated cytotoxicity (ADCC). First clinical trials using bispecific whole antibodies or chemically crosslinked Fab fragments [7–9] looked promising [10–13]. The strategy of bispecific miniantibodies might, however, also be useful for a wide range of related applications, such as the delivery of toxic proteins [14] and small molecule drugs such as radioisotopes [15,16].

We have chosen to create, as a model system, bispecific miniantibodies capable of binding to the EGF receptor (EGF-R) and CD2 at the same time. EGF-R is overexpressed by a wide range of tumors, whereas CD2 is expressed on cytotoxic T-cells (CTL) and natural killer cells (NK). Activation of CTL and NK close to tumor cells has previously been demonstrated by chemically crosslinked Fab fragments of the same antibodies from which our scFv are derived [17,18]. Activation of CTL and NK by the CD2 route is potentially very useful as it requires a second anti-CD2 monoclonal antibody [17,18] and thus avoids premature activation of the T-cells, which would give rise to high toxicity of the bispecific antibody. The parent monoclonal antibody for the anti-EGF-R scFv, 425, has already been tested in a clinical study in patients with advanced laryngeal and hypopharyngeal carcinomas [19].

Here we demonstrate that IgG $C_H1$ and κ-chain $C_L$ domains, fused to two scFv moieties, can be utilized to form heterodimers, when functionally expressed in *E. coli*. The advantage of this format is a longer reach to far apart antigens, compared to smaller bispecific variants, which is expected to be advantageous for bridging tumor cells with effector cells. In addition, the molecular size of six Ig domains has previously been shown to result in useful half-lives and good tumor-to-blood ratios [20]. Furthermore, the immunogenicity is expected to be low, since human sequences can be used for all components.

## 2. Materials and methods

### 2.1. Plasmid construction

An *Xba*I-*Hin*dIII cassette for dicistronic expression of two proteins was constructed by gene synthesis based on recursive PCR [21]. This cassette was cloned into a *Eco*RI- and *Eco*RV-free derivative of pASK30 [22] to give pKM30hetrop. In several PCR steps with intermediate subcloning into pCR-ScriptSK (Stratagene, USA) the anti-EGF-R scFv (named 425) [23], the anti-CD2 scFv (named M1) [18] (W. Strittmatter, unpublished), the IgG1 $C_H1$ domain including the first hinge cysteine [24,25], and the κ-chain $C_L$ domain [26,27] were cloned into this vector to give pKM30425M1ChCl. The $C_H1$ gene was amplified using the forward primer cg1_xho_hinge: TATCCTCGAG CCGAAGCCGT CTACTCCACC CGGGACTAGT GCAAAGACCA CTCCTCCG and the back primer cg1_nhe: TCTAGCTAGC ACCACCGCAG TCGCGCGGTA CG. The $C_L$ gene was amplified using the primers cl_sac: TGACGAGCTC CGCTGATGCT GCACCGAC and cl_his_hind: TCCCAAGCTT ACTAGTGATG GTGATGGTGA CCACCACACT CATTCCTGTT GAAG. In addition, a variant of the scFv M1 with a longer linker, named M1L, was constructed and cloned to give pKM30425M1LChCl. For comparison, the homodimeric miniantibody 425dhlx [4,23] with a hexa-histidine tail was also constructed to give the plasmid pKM30425dhlxh6. The scFv 425 in the bispecific construct and all homodimeric con-

*Corresponding author. Fax: (41) (1) 635-5712.
E-mail: plueckthun@biocfebs.unizh.ch

*Abbreviations:* Ab, antibody; 425, antibody binding to EGF-R; EGF-R, epidermal growth factor receptor (extracellular domain); ELISA, enzyme linked immunosorbent assay; IMAC, immobilized metal-ion affinity chromatography; M1, antibody binding to CD2; PCR, polymerase chain reaction; PDB, protein data bank; scFv, single-chain Fv antibody fragment; SDS sodium dodecyl sulfate; SPR, surface plasmon resonance


*PII* S0014-5793(98)00021-0

structs carry a short N-terminal FLAG tag [28]. All constructs obtained with PCR steps were confirmed by sequencing. The correct size of the proteins M1dhlx, M1Ldhlx, and 425dhlx was verified by electrospray ionization mass spectrometry.

### 2.2. Protein expression

Plasmids were transformed either into *E. coli* JM83 ($F^-$ *ara* Δ(*lac-proAB*) *rpsL* ($str^r$) [Φ*80dlac* Δ(*lacZ*)*M15*] *thi*) or BF18-61 (R. Wenderoth, unpublished), a RV308 (*lac74-gal*ISII::OP308-*strA*) derivative. Protein expression was carried out in LB broth in shake flasks, containing 0.1 g/l ampicillin. Overnight cultures were grown at 26°C and the main culture was inoculated to an $OD_{550}$ of 0.15 (typical dilution of 1:25–1:30) and grown at 25°C. Cells were induced at an OD of 0.5–0.6 and harvested by centrifugation at 5000×*g* after reaching a plateau phase, typically after 3–4 h at an OD of 2–2.5. Cell pellets were immediately frozen at −80°C and stored until use.

### 2.3. Protein purification

Cell pellets with miniantibody 425$C_H$-M1$C_L$ were thawed in a seven-fold volume of PBS (50 mM Na-phosphate, 150 mM NaCl) buffer, containing 10 mM imidazole and 1 mM $MgCl_2$, pH 7.4, and sonicated three times with continuous temperature monitoring in an ice-ethanol bath, between 3 and 8°C. To the lysates Tween-20 was added to a final concentration of 0.005% (v/v), and the sample was clarified by centrifugation at 49 000×*g* for 30 min, and filtered through 0.2 μm pores. The sample was applied to a $Ni^{2+}$-NTA column (Qiagen, Germany) with PBS-Tween as running buffer at 4°C, washed with 10 mM and 25 mM imidazole and eluted with 250 mM imidazole. The elution fraction was directly applied to an anti-425 idiotypic immunoaffinity column. After washing with PBS-Tween to baseline, samples were eluted with acidic buffer (100 mM glycine, 500 mM NaCl, and 0.005% Tween adjusted to pH 2.8 with HCl) into vials containing a predetermined amount of 1 M Tris base to adjust the eluate immediately back to pH 7.5. After purification samples were dialyzed against PBS-Tween. Miniantibody 425-$C_H$/M1-$C_L$ expressed in BF-18 was typically obtained at 125 μg/(l·$OD_{550}$) and miniantibody 425-$C_H$/M1L-$C_L$ expressed in JM83 at 20 μg/(l·$OD_{550}$).

### 2.4. SDS-PAGE, Western blot

Samples were analyzed by SDS-PAGE (12% minigels with 5% stacking gels) in sample buffer either with or without 100 mM DTT. Protein bands were quantitated from reducing samples by densitometry (Densitometer 300A and Imagequant software, Molecular Dynamics, USA). Immunoblots on nitrocellulose membranes were stained with Ponceau-S and protein bands marked. After blocking with 3% skim milk powder, proteins were detected, first using an anti-FLAG (Kodak, USA) or anti-His tag [29] monoclonal antibody, respectively, and second an anti-mouse Fc-peroxidase conjugate (Sigma, USA). The blot was developed with precipitating BM-blue (Boehringer Mannheim, Germany).

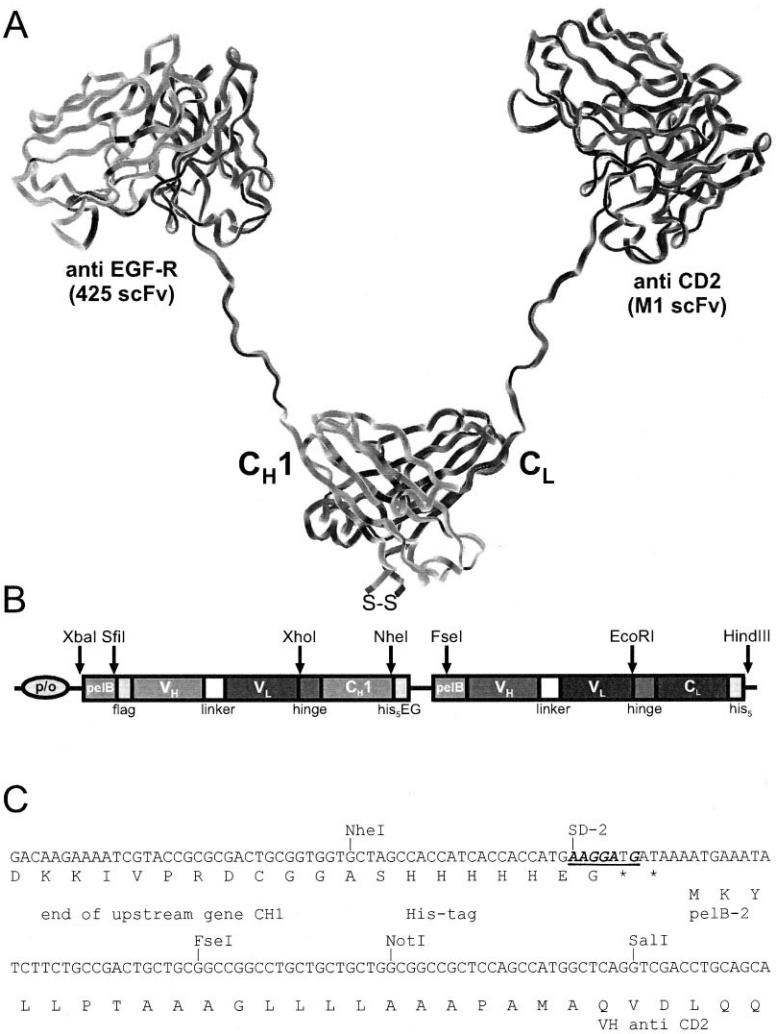


Fig. 1. Model of the protein structure (A), scheme of the gene arrangement (B), and detail of plasmid pKM30425M1ChCl (C) of the bispecific miniantibody. The model structure was assembled from minimized structures of a variable domain (PDB file 4FAB) with an attached linker, an extended modeled hinge region, and the constant domains $C_H1$ and $C_L$ (PDB file 1IKF). The gene sequence shows the C-terminal part of the first protein 425$C_H1$ including the Shine-Dalgarno (SD-2) sequence and the start of the second protein M1$C_L$ including the modified pelB signal sequence.

*2.5. Sandwich ELISA*

ELISA 96-well plates (Nunc Maxisorb, USA) were coated with 50 μl per well of 300 ng/ml CD2 extracellular domains (kindly provided by Drs. K. Willis, S. Simon, and A. van der Merwe) in PBS pH 7.4. After blocking with 3% skim milk powder, first the bispecific miniantibody sample was added, second, the anti-425 idiotypic monoclonal antibody, and third, the polyclonal anti-mouse Fc-peroxidase conjugate. The ELISA was developed with soluble BM-blue (Boehringer Mannheim).

*2.6. Size exclusion chromatography*

Samples of 30 μl were loaded onto a Superose-12 PC 3.2/30 column (Pharmacia, Sweden) equilibrated in PBS containing 0.005% Tween. Molecular weights were determined according to a linear regression of molecular weight standards (Sigma, USA) analyzed under the same conditions.

*2.7. Surface plasmon resonance biosensor*

Surface plasmon resonance biosensor measurements were performed with the BIAcore instrument (Biacore, Sweden). EGF-R (70 μl) at an approximate concentration of 0.2 μg/ml in 10 mM Na-acetate, pH 4.5 was coupled to a CM5 sensor chip (Biacore, Sweden) using standard EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide-HCl) NHS (*N*-hydroxysuccinimide) coupling chemistry with subsequent blocking by ethanolamine. The EGF receptor extracellular domain was purified by affinity chromatography from A431 cell culture supernatant (W. Strittmatter, unpublished data).

## 3. Results

*3.1. Construction of a cloning cassette for efficient polycistronic expression*

The translation efficiency of downstream proteins in polycistronic expression can be a problem if the spacing between the first and second coding sequence is too large [30]. Therefore, a cassette for dicistronic expression was designed with minimal spacing between the stop codon of the first and the start codon of the second gene. This was achieved by placing the Shine-Dalgarno sequence for the second protein within the coding sequence of the first protein as depicted in Fig. 1C. The Shine-Dalgarno sequence itself and the distance to the start codon were designed according to current knowledge [31–33]. To avoid read-through two stop codons were introduced, which do not interfere with the requirements for the nucleotides between the Shine-Dalgarno sequence and the protein translation start. The second translation product is also in a different frame, which should further promote reorientation of the ribosome. To avoid recombination between two similar pelB sequences and to facilitate cloning with unique restriction enzymes, the second pelB sequence was modified. A silent *Fse*I site was introduced, which cuts rarely in antibodies, as well as a mutation for a *Not*I site [34].

*3.2. Expression and assembly determined by SDS-PAGE, Western blot, and gel filtration*

As seen in the IMAC elution in Fig. 2A, scFv-M1$C_L$, encoded as a downstream gene in the dicistronic arrangement, was expressed at least to the same extent as the upstream scFv-425$C_H$. In contrast, when both proteins were expressed separately in the same homodimeric miniantibody format, scFv-M1 expressed significantly less functional protein than scFv-425 (data not shown). This is a strong indication that the designed polycistronic expression works well. After the anti-idiotypic affinity purification, specific for the scFv-425, 425$C_H$ is present in slight excess. Quantification of the intensity of the bands on the Coomassie stained gel indicates that about 63% heterodimer of the purified 425-$C_H$ is combined with the M1-$C_L$ after anti-idiotypic chromatography. As deduced from absorbance spectra, a typical 2 l shake flask culture using strain BF-18 yields in total about 800 μg protein after anti-scFv-425 immunoaffinity purification and dialysis, corresponding to approximately 500 μg bispecific miniantibody. The usage of strain JM83 results in slightly less protein. In the case of the miniantibody with a longer linker between $V_H$ and $V_L$ of M1, named scFv-M1L, the two scFv fusion proteins are difficult to distinguish on SDS-PAGE. The usage of the longer linker gave in general less pure protein.

The identity of protein bands after anti-idiotypic affinity chromatography was determined by immunoblot detection as seen in Fig. 2B,C. The 425-$C_H$ fusion contains a FLAG epitope and the M1-$C_L$ fusion a His tag, which can both be specifically detected. The anti-His tag monoclonal antibody solely recognizes C-terminal His tags, and therefore the 425-$C_H$ His tag, which is followed by two additional amino acids, is not detected. In a reducing SDS-PAGE, both bands are resolved, whereas in an oxidizing gel both antibodies detect the same band at a molecular weight corresponding to a heterodimer, indicating that it contains both species. A slight

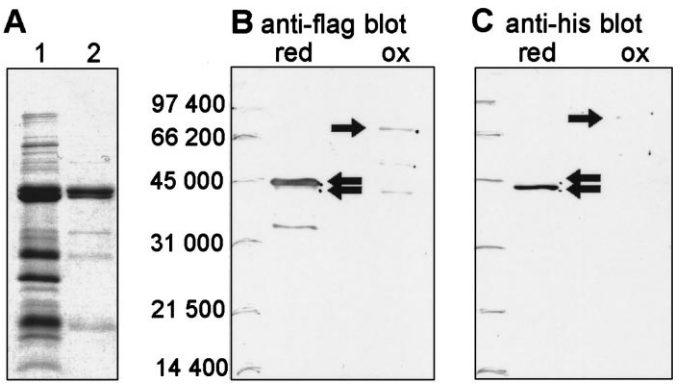


Fig. 2. Identification of $C_HC_L$ miniantibody protein bands. A: Coomassie stained SDS gel of the purification of the bispecific miniantibody 425$C_H$M1$C_L$ after IMAC (1) and after anti-idiotypic affinity chromatography (2). B, C: Western blot of the 425$C_H$M1$C_L$ miniantibody. Two blots with the same sample were stained with either anti-FLAG (B) or anti-His tag monoclonal antibody (C). In both cases the bispecific miniantibody was loaded under reducing conditions (red) and under oxidizing conditions (ox). Under reducing conditions the two proteins forming the miniantibody dissociate and are stained separately: the anti-FLAG Ab detects 425-$C_H$ and the anti-His tag Ab detects M1-$C_L$. Under oxidizing conditions both antibodies detect the same band at the molecular weight of the heterodimer. Protein bands were marked in a Ponceau-S stain prior to immunostaining.

degradation band can also be seen. The 425-$C_H$ chain comprises 372 residues with a molecular weight of 39 636 Da (calculated without disulfide bridges), and the M1-$C_L$ 364 residues with 39 819 Da.

Size exclusion chromatography revealed a molecular mass of 87 kDa for the $C_HC_L$ miniantibody, in good agreement with the calculated mass of 79.5 kDa. For comparison, the homodimeric miniantibody 425dhlx was measured with a molecular weight of 77 kDa and for the M1dhlx with a molecular weight of 75 kDa, both also in good agreement with the calculated masses of 67 kDa and 66 kDa, respectively.

### 3.3. Bispecificity detected in ELISA

Bispecific binding was analyzed in a sandwich ELISA. After coating with CD2, the ELISA plate was incubated first with the bispecific miniantibody, second with a monoclonal anti-idiotypic antibody recognizing the EGF receptor specificity, and third for detection with a peroxidase-conjugated polyclonal mouse Fc-specific serum. The signal at 200 nM miniantibody could be inhibited by co-incubation with either 1 μM CD2 or 1 μM EGF-R, indicating that binding is specific on both sides Fig. 3.

### 3.4. EGF receptor binding measured with surface plasmon resonance

The functionality of the anti-EGF receptor scFv was further tested using the BIAcore instrument. The experiment was designed to detect possible loss in avidity when switching from the natural bivalent binding of a monoclonal antibody to the monovalent binding in the bispecific miniantibody format. Therefore, EGF-R was coupled to the sensor chip at a high coating density of 1600 resonance units (RU). Indeed, the homodimeric miniantibody scFv-425dhlx (Fig. 4B) fully reproduces the binding characteristics of the monoclonal antibody (Fig. 4C), whereas the $C_HC_L$ miniantibody, which binds EGF-R only monovalently, displays a faster off-rate (Fig. 4A). Avidity is difficult to express in exact biophysical terms, since it depends on molecular size and ligand density [5,6]. However, using the same surface and molecular concentrations of analyte, direct comparisons can be made. The differences seen in the signal intensity reflect the differences in the molecular size (425-$C_H$/M1-$C_L$ 79 kDa, 425dhlx 67 kDa, mAb 150 kDa), and the higher off-rate in the case of the bispecific miniantibody.

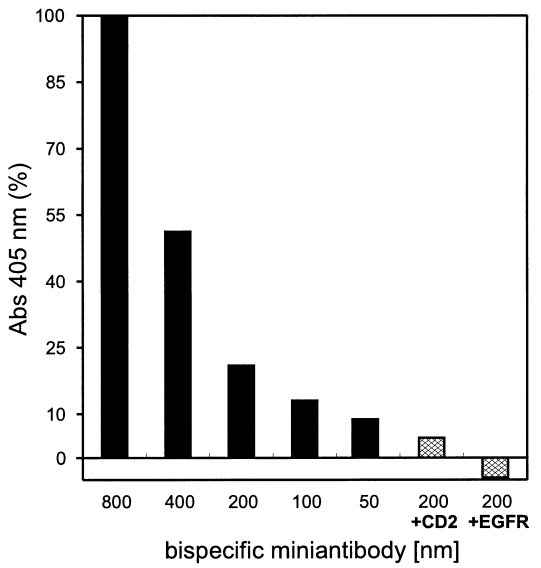


Fig. 3. Sandwich ELISA for the analysis of bispecificity. CD2 was coated, and the ELISA plate was incubated with bispecific miniantibody 425$C_H$(anti-EGF-R)-M1$C_L$(anti-CD2). Anti-EGF-R specificity was detected with an anti-idiotypic monoclonal antibody directed against the 425 scFv. Inhibition with 1 μM CD2 and 1 μM EGF-R demonstrates specificity. The secondary antibody was a peroxidase conjugated anti-Fc specific polyclonal serum. The blank value without bispecific miniantibody was subtracted from all measurements.

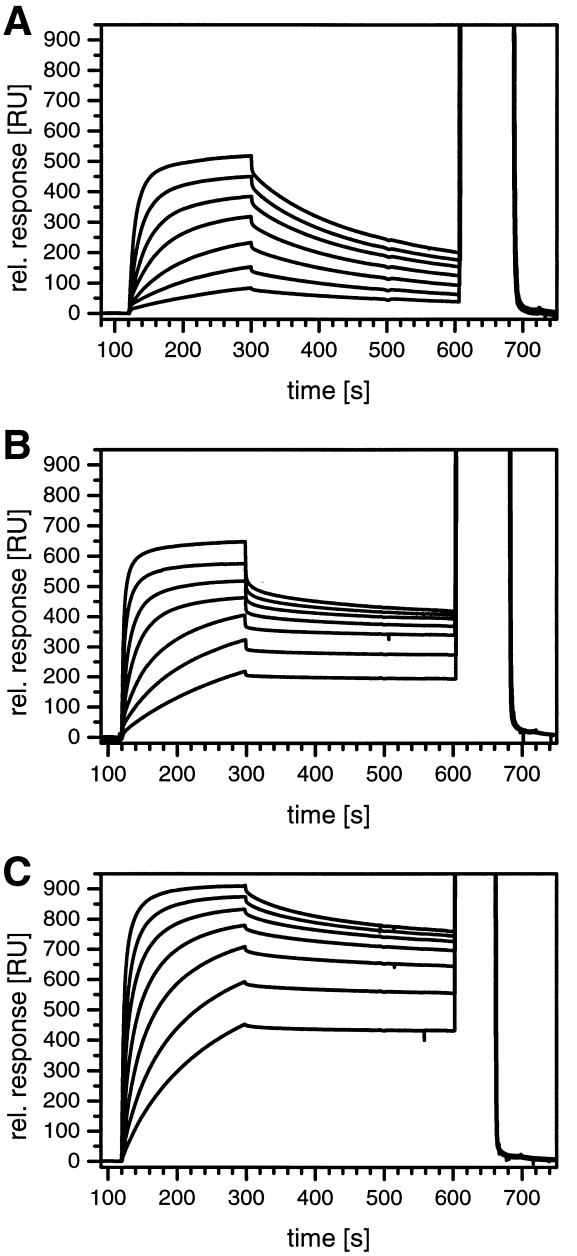


Fig. 4. BIAcore sensorgrams of EGF-R binding. A dilution series of miniantibody 425$C_H$-M1$C_L$ (A), homobivalent miniantibody 425-dhlx (B) and the parental monoclonal antibody (C). Binding to a surface coated with 1600 RU EGF-R was measured at concentrations of 25, 50, 100, 200, 400, 800, and 1600 nM for each molecule.

## 4. Discussion

The results presented demonstrate that functionally bispecific miniantibodies, heterodimerized via $C_H1$ and $C_L$, can be obtained in functional form from *E. coli*. It was shown that such complex molecules comprising heterodimers of three different Ig domains, each with disulfide linkages between them, can be functionally formed in *E. coli*. A new polycistronic expression cassette was successfully employed for this purpose.

Several other techniques have been developed to combine two different antibody binding sites in one molecule [6]. Conceptually, the simplest form of bringing two specificities together is a polypeptide linker between two scFv [35,36] or Fab fragments [37]. Another minimal size antibody derivative is the diabody [38], with two crosswise paired scFv, which are, however, very restricted in reach. Simple heterodimerization domains fused to scFv like the Jun/Fos coiled-coil lead to a mixture of homo- and heterodimers [39] and need to be assembled by refolding [40]. Reengineering of the $C_H3$ domain into a heterodimerization domain [41] was shown to be capable of forming antibody immunoadhesin hybrids and may also be useful for heteromerization. A large molecule is the result of the fusion of scFv fragments to whole antibodies serving as dimerization devices [42]. The $C_HC_L$ miniantibody strategy described here leads to the desired assembly with a small size but a long reach.

The anti-EGF-R scFv-425 and the anti-CD2 scFv-M1 were constructed from monoclonal antibodies without further mutations. The scFv-425dhlx, homodimerized via a helix-turn-helix module, expresses well [23], whereas the homodimeric M1dhlx causes expression problems. Taking this into account, the robustness and yield of about 125 μg/(l·OD) of a simple $C_HC_L$ miniantibody shake flask culture is encouraging and invites scFv optimization. The reasons for the lower final yields with the scFv-M1L with a longer linker, which are also seen in the homodimeric construct (data not shown), are most likely due to an increased toxic effect which limits the cell growth. This is even more pronounced in strain JM83.

The fact that the designed dicistronic expression cassette presented here appears to be overachieving in terms of the functional yield of the downstream protein can most likely be attributed to differences in folding and membrane translocation efficiency of the $C_H1$ and $C_L$ domains. However, the simultaneous coding of both proteins on one plasmid offers the possibility of reproducible fine tuning.

The scFv-M1, regardless of whether it is expressed as a heterodimeric or homodimeric miniantibody, does not reach the slow off-rate of its parental monoclonal antibody (data not shown). This accounts for the low signals seen in sandwich ELISA, since in this assay two additional incubation steps were used. In contrast, the homodimeric scFv-425 has the same binding characteristics as the monoclonal antibody as seen in BIAcore. The bispecific miniantibody is only monovalent for each specificity. Based on previous experiments with Fab fragments chemically coupled to form heterodimers [18], which showed antibody-dependent cell killing, monomeric binding in the presented case appears to be sufficient for the application of the miniantibody.

The implementation of two optimization strategies is likely to increase yields dramatically. First, the engineering of the scFv molecule for improving their folding characteristics [43–45] will ensure that scFv from well analyzed monoclonal antibodies or natural libraries can be further improved to meet the protein sequence criteria for high level expression in *E. coli*. Second, the knobs into holes engineering approach [41] is an attractive perspective to further optimize the heteromerization of $C_H1$-$C_L$ as well as the specific formation of the scFv itself. In conclusion, the use of $C_H1$ and $C_L$ as heterodimerization tool appears to be a promising strategy for generating heterodimeric proteins.

*Acknowledgements:* This work was supported by the Deutsche Bundesministerium für Bildung und Forschung. The authors thank Drs. Simon Davis and Anton van der Merwe (Oxford University, UK) and Dr. Kevin Willis (Procept, Cambridge, MA, USA) for providing purified CD2 and Dr. P. Gehrig for mass spectrometry measurements.

## References

[1] Huston, J.S. et al. (1988) Proc. Natl. Acad. Sci. USA 85, 5879–5883.
[2] Bird, R.E. et al. (1988) Science 242, 423–426.
[3] Glockshuber, R., Malia, M., Pfitzinger, I. and Plückthun, A. (1990) Biochemistry 29, 1362–1367.
[4] Pack, P. and Plückthun, A. (1992) Biochemistry 31, 1579–1584.
[5] Pack, P., Müller, K., Zahn, R. and Plückthun, A. (1995) J. Mol. Biol. 246, 28–34.
[6] Plückthun, A. and Pack, P. (1997) Immunotechnology 3, 83–105.
[7] Milstein, C. and Cuello, A.C. (1983) Nature 305, 537–540.
[8] Karpovsky, B., Titus, J.A., Stephany, D.A. and Segal, D.M. (1984) J. Exp. Med. 160, 1686–1701.
[9] Perez, P., Hoffman, R.W., Shaw, S., Bluestone, J.A. and Segal, D.M. (1985) Nature 316, 354–356.
[10] Canevari, S. et al. (1995) J. Natl. Cancer Inst. 87, 1463–1469.
[11] Kroesen, B.J., Buter, J., Sleijfer, D.T., Janssen, R.A., van der Graaf, W.T., The, T.H., de Leij, L. and Mulder, N.H. (1994) Br. J. Cancer 70, 652–661.
[12] Valone, F.H. et al. (1995) J. Clin. Oncol. 13, 2281–2292.
[13] Weiner, L.M., Clark, J.I., Davey, M., Li, W.S., Depalazzo, I.G., Ring, D.B. and Alpaugh, R.K. (1995) Cancer Res. 55, 4586–4593.
[14] Bonardi, M.A., Bell, A., French, R.R., Gromo, G., Hamblin, T., Modena, D., Tutt, A.L. and Glennie, M.J. (1992) Int. J. Cancer Suppl. 7, 73–77.
[15] Le Doussal, J.M. et al. (1993) J. Nucl. Med. 34, 1662–1671.
[16] Stickney, D.R., Anderson, L.D., Slater, J.B., Ahlem, C.N., Kirk, G.A., Schweighardt, S.A. and Frincke, J.M. (1991) Cancer Res. 51, 6650–6655.
[17] Meuer, S.C. et al. (1984) Cell 36, 897–906.
[18] Wild, M. (1995) PhD Thesis, Universität Karlsruhe.
[19] Bier, H., Reiffen, K.A., Haas, I. and Stasiecki, P. (1995) Eur. Arch. Otorhinolaryngol. 252, 433–439.
[20] Hu, S., Shively, L., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y., Shively, J.E. and Wu, A.M. (1996) Cancer Res. 56, 3055–3061.
[21] Prodromou, C. and Pearl, L.H. (1992) Protein Eng. 5, 827–829.
[22] Skerra, A. and Plückthun, A. (1988) Science 240, 1038–1041.
[23] Horn, U. et al. (1996) Appl. Microbiol. Biotechnol. 46, 524–532.
[24] Honjo, T., Obata, M., Yamawaki-Katoaka, Y., Kataoka, T., Kawakami, T., Takahashi, N. and Mano, Y. (1979) Cell 18, 559–568.
[25] Skerra, A. and Plückthun, A. (1991) Protein Eng. 4, 971–979.
[26] Altenburger, W., Neumaier, P.S., Steinmetz, M. and Zachau, H.G. (1981) Nucleic Acids Res. 9, 971–981.
[27] Plückthun, A. and Skerra, A. (1989) Methods Enzymol. 178, 497–515.
[28] Knappik, A. and Plückthun, A. (1994) BioTechniques 17, 754–761.
[29] Lindner, P., Bauer, K., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R. and Plückthun, A. (1997) BioTechniques 22, 140–149.
[30] Schoner, B.E., Belagaje, R.M. and Schoner, R.G. (1990) Methods Enzymol. 185, 94–103.

[31] Chen, H., Bjerknes, M., Kumar, R. and Jay, E. (1994) Nucleic Acids Res. 22, 4953–4957.
[32] Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G.D. and Gold, L. (1992) Mol. Microbiol. 6, 1219–1229.
[33] Barrick, D., Villanueba, K., Childs, J., Kalil, R., Schneider, T.D., Lawrence, C.E., Gold, L. and Stormo, G.D. (1994) Nucleic Acids Res. 22, 1287–1295.
[34] Söderlind, E., Lagerkvist, A.C., Duenas, M., Malmborg, A.C., Ayala, M., Danielsson, L. and Borrebaeck, C.A. (1993) Biotechnology (NY) 11, 503–507.
[35] Mallender, W.D. and Voss Jr., E.W. (1994) J. Biol. Chem. 269, 199–206.
[36] Mack, M., Riethmüller, G. and Kufer, P. (1995) Proc. Natl. Acad. Sci. USA 92, 7021–7025.
[37] Zapata, G., Ridgway, J.B.B., Mordenti, J., Osaka, G., Wong, W.L.T., Bennett, G.L. and Carter, P. (1995) Protein Eng. 8, 1057–1062.
[38] Holliger, P., Prospero, T. and Winter, G. (1993) Proc. Natl. Acad. Sci. USA 90, 6444–6448.
[39] Pack, P. (1994) PhD Thesis, Ludwig-Maximilians-Universität, Munich.
[40] de Kruif, J. and Logtenberg, T. (1996) J. Biol. Chem. 271, 7630–7634.
[41] Ridgway, J.B., Presta, L.G. and Carter, P. (1996) Protein Eng. 9, 617–621.
[42] Coloma, M.J. and Morrison, S.L. (1997) Nat. Biotechnol. 15, 159–163.
[43] Knappik, A. and Plückthun, A. (1995) Protein Eng. 8, 81–89.
[44] Nieba, L., Honegger, A., Krebber, C. and Plückthun, A. (1997) Protein Eng. 10, 435–444.
[45] Jung, S. and Plückthun, A. (1997) Protein Eng. 10, 959–966.