Bioseparation **9:** 179–183, 2000.
© 2000 *Kluwer Academic Publishers. Printed in the Netherlands.*

Efficient heterodimerization of recombinant bi- and trispecific antibodies

R. Schoonjans, A. Willems, J. Grooten & N. Mertens*
Molecular Immunology Unit, VIB1 – Department of Molecular Biology, University of Gent, K.L.Ledeganckstraat 35, B-9000 Gent, Belgium, Tel.: ++-32-(0)9-264 51 34, Fax: ++-32-(0)9-264 51 34
E-mail: Nico.Mertens@DMB.RUG.AC.BE

Received 31 August 1999; accepted in revised form 5 January 2000

Key words: bispecific antibody, cancer iummunotherapy, heterodimerization, immunoconjugate, recombinant gene expression

Abstract

Bispecific antibodies (BsAb) are promising therapeutic tools in tomorrow's medicine. Expression systems favoring efficient heterodimerization of intermediate-sized bispecific antibodies will significantly improve existing production methods. By C-terminal fusion of scFv molecules to the Fd- and the L-chains efficient heterodimerization in mammalian cells was obtained and a novel intermediate sized, disulfide stabilized BsAb could be efficiently produced. This type of antibody derivative easily allows for the production of trispecific antibodies, BsAb with bivalent binding for one antigen, or immunoconjugates.

Introduction

Bispecific antibodies (BsAb) are versatile tools in the development of new, experimental therapies of various diseases. Typically, one part of the BsAb specifically recognizes a target molecule or cell (e.g. a cancer cell), while the other part may be directed towards an enzyme, a toxin, a virus or an effector cell (e.g. a cytotoxic T cell). The problem of producing functional BsAb in sufficient quantity and with high purity is still hampering their general use in clinical applications. Production of bispecific antibodies is usually achieved by either chemical crosslinking of Fab' fragments or by the hybrid hybridoma technique. This however requires extensive post-production purification steps to isolate the bispecific moiety from all by-products. Recombinant DNA methodology and antibody engineering techniques have already contributed in facilitated production in bacterial expression systems (Holliger et al., 1993; Horn et al., 1996; Zhu et al., 1996). scFv heterodimers have been produced by direct genetic fusion (Mack et al., 1995), or linked with a helical dimerization domain (Pack and Pluckthun, 1992; Luo et al., 1997). These heterogeneous heterodimerization extensions, however, increase the possibility of immunogenicity. Besides the possible immunogenicity of the heterologous heterodimerization extensions, these molecules are generally cleared too rapidly from the body to allow efficient accumulation at the tumor site, due to their small size (Milenic et al., 1991). It was shown that an intermediate sized molecule compromises between efficient tissue penetration while avoiding rapid body clearance in the kidney (Hu et al., 1996). The heterodimerization of CL and its natural partner CH1 has been proposed to drive the heterodimerization of scFv molecules (Muller et al., 1998). This approach offers several advantages. First of all, the natural heterodimeric interaction between CL and CH1 circumvents the need for protein engineering (e.g. "knobs into holes" Ridgway et al., 1996) to achieve complementarity. Second, a single, natural disulphide bridge stabilizes the CL-CH1 heterodimer. Production in mammalian cells of CL-CH1 heterodimers should enhance on the efficiency and specificity of heterodimerization, since this is presumed to be enhanced by the chaperoning of the heavy chain-binding protein (BiP) to newly formed CH1 domain the endoplasmic reticulum (Hendershot et al., 1987). BiP is displaced after expression of the light chain (L), by

* Corresponding author

proper CL-CH1 interaction. Production of CL-CH1 minibodies in the absence of BiP in *Escherichia coli*, resulted in only 63% of heterodimerization (Muller et al., 1998).

We here report secretion in mammalian cells to be more efficient if in addition to the CL-CH1 interaction, also the VL-VH interaction could contribute to displace BiP. We further propose a model for bispecific antibodies based on the fusion of a scFv to the C-terminus of the Fab heavy chain. This results in a bispecific molecule of intermediate size (~80 kDa), which is formed by preferential heterodimerization of the L chain with the Fd-scFv fusion. In this model the C-terminal fusion of the scFv to the Fab moiety offers a longer reach for antigen, what is expected to be advantageous for bridging target cells with effector cells. Trifunctional molecules (110 kDa) could be created by also extending the L-chain with a scFv or another type of molecule. We believe this model to be a good candidate for production of bipecific antibodies and derivatives in mammalian cells, either *in vitro* or *in vivo*.

Material and methods

Cell lines

HEK293T, a human embryonic kidney cell line transfected with SV40 large T-antigen (SV40T tsA1609) (DuBridge et al., 1987) was used for transient eukaryotic expression. SP2/0-Ag14 are non-Ig secreting myeloma cells. TE2 cells are murine, CD3 positive Th1-type T-cells (Grooten and Fiers, 1989). MO4I4 cells are MO4mouse fibrosarcoma cells transfected with the hPLAP gene (Hendrix et al., 1991; Smans et al., 1995). BCL1^{vitro} cells are myeloma cells expressing the BCL1 IgM ideotype antigen and adapted for in vitro passage. All cells were cultured as described, all medium components were from GibcoBRL (UK).

Plasmids and gene assembly

Restriction enzymes and DNA modifying enzymes and polymerases were used as recommended by the manufacturers. DNA amplification was performed with Vent-DNA polymerase (New England Biolabs, MA). E6, B1 and 2c11 denote the genes or gene fragments of an αhPLAP, an αBCL1 and an αmCD3 (De Jonge et al., 1995) monoclonal antibody respectively. Expression plasmids were constructed in pCAGGS (Niwa et al., 1991). The cloning of the light of the parental αhPLAP moAb E6 (IgG2b/κ) in the vector pSV51E6L has been described previously (De Sutter et al., 1992). The E6Fd fragment encodes VH, CH1 and the first five amino acids (not containing cysteines) of the upper hinge region (EPSGP). Fusion with CH1 or CL was carried out before the ‘elbow’ region of the Fab chains (EMKRAD and SAAKTT in the L and Fd chains respectively). Gene assembly was carried out by introduction of suitable restriction sites using modifying PCR primers. All PCR-derived fragments were sequence verified after cloning.

Production and purification of recombinant antibody fragments

For transient expression, HEK293T cells were transfected according to the $Ca_3(PO_4)_2$ precipitation method (O’Mahoney and Adams, 1994). The cells were covered with supplemented DMEM containing 5 mg/l bovine insulin, 5 mg/l transferrin and 5 μg/l selenium (ITS) replacing the FCS. Medium was harvested every 48 h after transfection.

For stable expression lines, SP2/0-Ag14 cells were electroporated, cultured in selective medium, subcloned and screened for production. The secreted Fab-scFv-$(His)_6$ protein was purified under native conditions from the culture supernatant using immobilised metal affinity chromatography (IMAC). Finally, the protein was dialysed to PBS and stored at 4 °C. Gel filtration was performed on a calibrated Sephacryl S300 column (Amersham Pharmacia Biotech).

T-cell proliferation assay

For the MO4I4 fibrosarcoma cells or the BCL1^{vitro} lymphoma cells spleen derived T-cells from syngenic C3H/HeOUico or BALB/c mice receptively were used. All mice were purchased from the Charles River group (Sulzfeld, DE). MO4I4 and BCL1^{vitro} tumor cells were pre-treated with 50 μg/ml mitomycin C at 37 °C in the dark for 12h and 1.5h respectively. After removal of the mitomycin C, 5 $\times$ 10^5 treated cells were co-cultured with 1 x 10^5 splenic T-cells in a round bottom well in the presence of the indicated concentration of the bispecific Fab-scFv BsAb. After 48 h, the T-cells were pulsed with 0,5 μCi of tritium-thymidine ([^{3}H]TdR, 1mCi/ml, Amersham Pharmacia Biotech). 18 h later the cells were disrupted by freeze-thawing, the DNA was spotted on a filter and washed and the incorporated radioactivity was measured by scintillation counting (Top-Count; Packard, CO, USA).

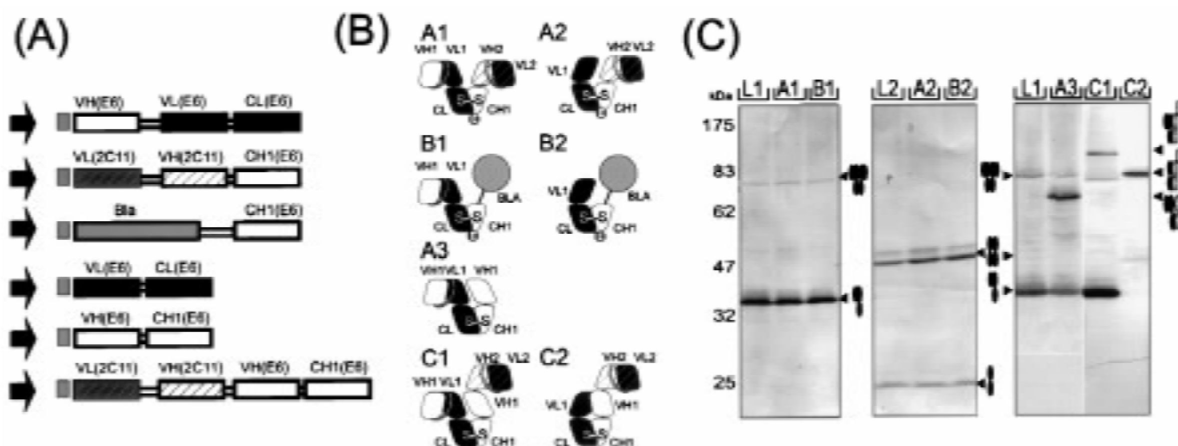

Figure 1. CL and CH1 need extension with VL and VH domains to be secreted in mammalian cells. Schematic representation of the expression vector gene inserts (A) and the expected proteins produced (B). (C) Western Blot analysis of the culture supernatant revealed by anti mouse IgG serum.

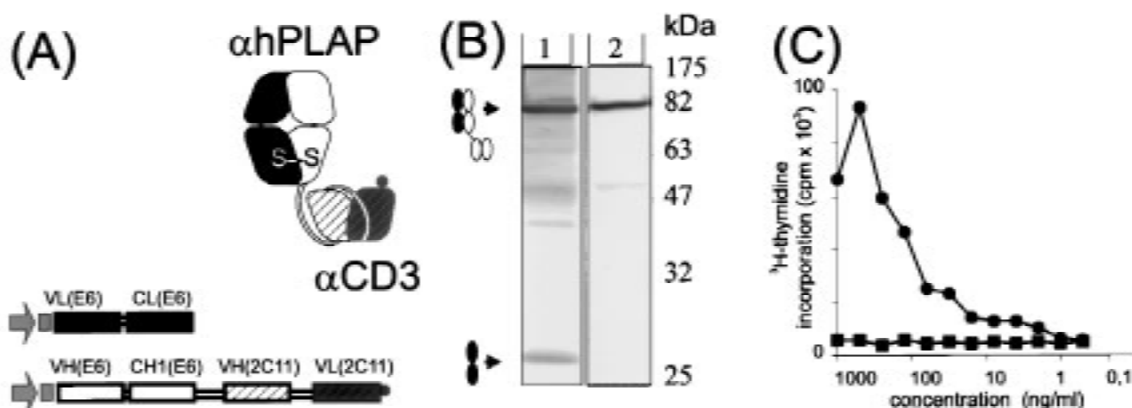

Figure 2. Functional expression of a Fab-scFv fusion as a BsAb. (A) Schematic representation of the gene fusion and of the expected protein formed. (B) Western Blot analysis of culture supernatant by 1) anti mouse IgG or 2) by incubation with hPLAP. (C) T-proliferation in the presence of hPLAP+ MO4I4 cells and a specific (circle) or a non-specific (square) BsAb in the Fab-scFv format.

Results and discussion

Heterodimerization by CL-CH1 interaction in eukaryotic cells can depend on appropriate VL-VH pairing

In a first attempt, minibodies were constructed using the CL and CH1 domain on their own to promote heterodimerization of two different scFv molecules fused to their N-terminus (Muller et al., 1998). However, after cotransfection of expression plasmids for the scFv(αCD3)-CL and the scFv(αhPLAP)-CH1 fusion proteins, largely all produced protein was in the monomer format. To eliminate any possible steric hindrance caused by the fusion of the scFv molecules to the CL and CH1 domains, a derivative was made with a longer flexible linker separating the fusion partner from CH1. For simplifying the analysis of the construct we used a β-lactamase molecule (bla) as a fusion partner, which allows for detection of heterodimers simply on the basis of molecular weight. When co-expressing the bla-CH1 fusion with the scFv(αCD3)-CL fusion, again only CL-containing products could be found in the medium. This is especially remarkable since this was also true when the bla-CH1-E fusion was co-expressed with a native L chain. The L-chains can be expressed on their own and appear both as monomers and as homodimers, so they can be functionally expressed without association to any other partner. However, in the reversed situation when a scFv(αCD3)-CL fusion was co-expressed with

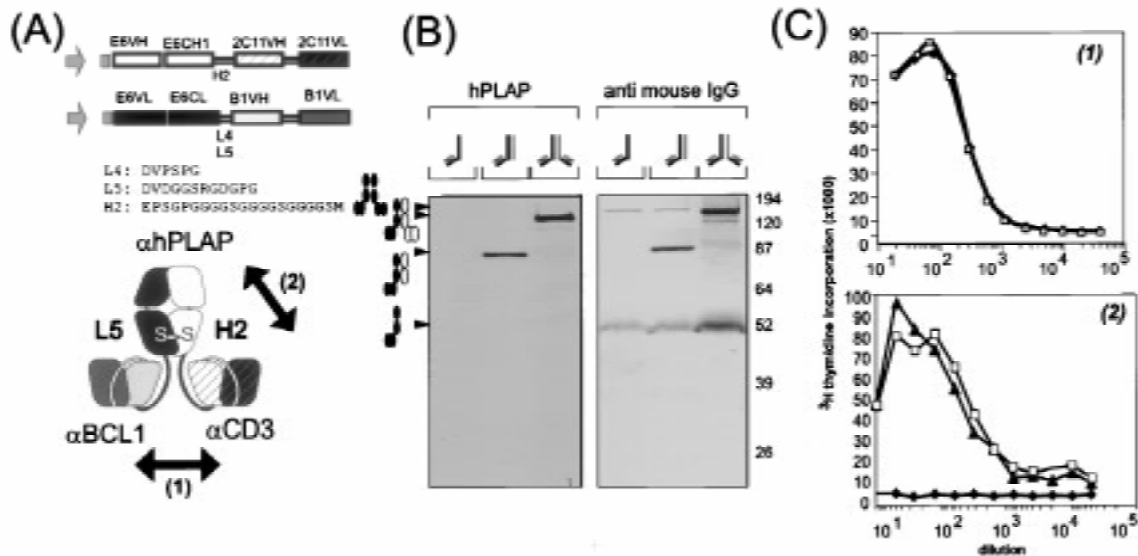

Figure 3. Expression and functionality of trispecific antibodies. (A) Schematic representation of the expression vector fusion genes and the expected protein to be formed. (B) Expression detected with hPLAP or with anti mouse IgG serum. (C) T-cell proliferation assay with BCL1 (1) or hPLAP$^+$ fibrosarcoma cells (2) as targets.

a native Fd chain, a scFv(αCD3)-CL:Fd heterodimer could be formed (Figure 1, A3), even when the (complete) Fd chain was N-terminally extended with another scFv (Figure 1, C1). A more efficient heterodimerization however was observed when the scFv-Fd fusion was co-expressed with the native L chain (Figure 1, C2). Heterodimerization apparently depends on the constraints posed upon the VH(E6) domain to pair with the VL(E6) domain to form a Fab(E6) chain. Apparently, only structures with the possibility of forming a Fab chain give rise to heterodimers upon expression in mammalian cells.

Fab-scFv fusion molecule as a model system for intermediate sized BsAb production

We wanted to investigate whether the Fab-context could be used as a heterodimeric scaffold to build bispecific antibodies. To allow for maximal range of antigen reach, we elongated the Fd(αhPLAP) chain at its C-terminus with a flexible peptide linker and the sequence coding for the scFv(αCD3). The glycine-serine rich peptide linker $(G_4S)_3$ was used to connect both subunits. When transiently co-expressed in HEK293T cells, the plasmid-sets gave rise to the expected (αhPLAP x αCD3) Fab-scFv molecule. As seen on the Western blot developed with anti-mouse IgG, the selective formation of heterodimers was very efficient and there were only a few minor degradation products and some free light chains (Figure 2). The Fab(E6)-scFv(2c11) BsAb was found to heterodimerize very efficiently: up to 90% of all secreted immunoglobulin was in the heterodimeric format. Typically, 1 mg of pure bispecific Fab-scFv could be obtained from 1,5 l culture medium by transient expression. Stable transformed SP2/0 cell lines produced up to ten fold as much. A final gel filtration step on a calibrated gel filtration column showed that the Fab-scFv BsAb was soluble as a monomer (data not shown). The conservation of functional binding specificity of the constituting components of the Fab(E6)-scFv(2c11) BsAb was confirmed with flow-cytometric analysis of binding to the respective target cells. A cellular ELISA confirmed binding of hPLAP while bound to T-cells. The Fab-scFv molecule showed to be able to crosslink two different cells by induction of T-cell proliferation in the presence of antigen expressing target cells (Figure 2).

Fd:L mediated heterodimerization of two scFv molecules: efficient expression of trispecific molecules

Analogous to the L:Fd-scFv heterodimerization, we were also able to obtain efficient L-scFv:Fd heterodimerization. Next, both the L-scFv and the Fd-scFv fusion molecules were shown to be able to heterodimerize as the major immunoglobulin derived product formed in the culture supernatant. As a model system

we used three different specificities: two tumor markers (αhPLAP and αBCL1) and an αCD3 specificity to capture T-cells. To connect the scFv to the L chain we examined two different peptide linkers of 6 and 12 amino acids long. Both linkers gave efficient heterodimerization of the L:Fd heterodimerized products. ELISA and flow cytometry showed functional binding of all three specificities to their respective target cells. T-cell proliferation could be induced with the (αhPLAP $\times$ αBCL1 $\times$ αCD3) trispecific antibody in the presence of BCL1-lymfoma cells or in the presence of the hPLAP$^+$ MO4I4 cells, showing that the molecule was able to crosslink two different cells along the (αhPLAP x αCD3) axis and the (αBCL1 x αCD3) axis. Both the 6 and the 12 amino acid linker performed equally well in this assay (Figure 3).

In a similar way it was possible to express trifunctional bispecific molecules, with a bivalent binding towards one target cell. Also trivalent binding antibody derivatives could be produced. Upon replacement of one of the scFv molecules with another type of molecule, such as IL2 to enhance immunostimulation, also a bispecific binding immunoconjugate could be produced, showing the versatility of the BsAb design (data not shown).

References

Brissinck J, Demanet C, Moser M, Leo O and Thielemans K. (1991) Treatment of mice bearing BCL1 lymphoma with bispecific antibodies. J Immunol 147: 4019–4026

De Jonge J, Brissinck J, Heirman C, Demanet C, Leo O, Moser M and Thielemans K. (1995) Production and characterization of bispecific single-chain antibody fragments. Mol Immunol 32: 1405–1412

De Sutter K, Feys V, Van de Voorde A and Fiers W. (1992) Production of functionally active murine and murine::human chimeric F(ab')2 fragments in COS-1 cells. Gene 113: 223–230

DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH and Calos MP. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 7: 379–87

Grooten J and Fiers W. (1989) Acquisition by the murine host of responsiveness toward various neoplastic cell lines, but not toward self, through adoptive transfer of a helper T-lymphocyte clone with antiself specificity. Cancer Res 49: 3872–8

Hendershot L, Bole D, Kohler G and Kearney JF. (1987) Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. J Cell Biol 104: 761–7

Hendrix PG, Dauwe SE, Van de Voorde A, Nouwen EJ, Hoylaerts MF and De Broe ME. (1991) Radiolocalisation and imaging of stably HPLAP-transfected MO4 tumours with monoclonal antibodies and fragments. Br J Cancer 64: 1060–1068

Holliger P, Prospero T and Winter G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. Proc Natl Acad Sci USA 90: 6444–6448

Horn U, Strittmatter W, Krebber A, Knupfer U, Kujau M, Wenderoth R, Muller K, Matzku S, Pluckthun A and Riesenberg D. (1996) High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high- cell-density fermentation under non-limited growth conditions. Appl Microbiol Biotechnol 46: 524–532

Hu S, Shively L, Raubitschek A, Sherman M, Williams LE, Wong JY, Shively JE and Wu AM. (1996) Minibody: A novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. Cancer Res 56: 3055–61

Luo D, Geng M, Noujaim AA and Madiyalakan R. (1997) An engineered bivalent single-chain antibody fragment that increases antigen binding activity. J Biochem (Tokyo) 121: 831–4

Mack M, Riethmuller G and Kufer P. (1995) A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. Proc Natl Acad Sci USA 92: 7021–7025

Milenic DE, Yokota T, Filpula DR, Finkelman MA, Dodd SW, Wood JF, Whitlow M, Snoy P and Schlom J. (1991) Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. Cancer Res 51: 6363–71

Muller KM, Arndt KM, Strittmatter W and Pluckthun A. (1998) The first constant domain (C(H)1 and C(L)) of an antibody used as heterodimerization domain for bispecific miniantibodies. FEBS Lett 422: 259–64

Niwa H, Yamamura K and Miyazaki J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108: 193–9

O'Mahoney JV and Adams TE. (1994) Optimization of experimental variables influencing reporter gene expression in hepatoma cells following calcium phosphate transfection. DNA Cell Biol 13: 1227–32

Pack P and Pluckthun A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric FV fragments with high avidity in *Escherichia coli*. Biochemistry 31: 1579–1584

Ridgway JB, Presta LG and Carter P. (1996) 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 9: 617–621

Smans KA, Hoylaerts MF, Narisawa S, Millan JL and De Broe ME. (1995) Bispecific antibody-mediated lysis of placental and germ cell alkaline phosphatase targeted solid tumors in immunocompetent mice. Cancer Res 55: 4383–4390

Zhu Z, Zapata G, Shalaby R, Snedecor B, Chen H and Carter P. (1996) High level secretion of a humanized bispecific diabody from *Escherichia coli*. Biotechnology (N Y) 14: 192–6