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Fab Chains As an Efficient Heterodimerization Scaffold for the Production of Recombinant Bispecific and Trispecific Antibody Derivatives

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Due to their multispecificity and versatility, bispecific Abs (BsAbs) are promising therapeutic tools in tomorrow’s medicine. Especially intermediate-sized BsAbs that combine body retention with tissue penetration are valuable for therapy but necessitate expression systems that favor heterodimerization of the binding sites for large-scale application. To identify heterodimerization domains to which single-chain variable fragments (scFv) can be fused, we compared the efficiency of heterodimerization of CL and CH1 constant domains with complete L and Fd chains in mammalian cells. We found that the isolated CL:CH1 domain interaction was inefficient for secretion of heterodimers. However, when the complete L and Fd chains were used, secretion of L:Fd heterodimers was highly successful. Because these Fab chains contribute a binding moiety, C-terminal fusion of a scFv molecule to the L and/or Fd chains generated BsAbs or trispecific Abs (TsAbs) of intermediate size (75–100 kDa). These disulfide-stabilized bispecific Fab-scFv (“bibody”) and trispecific Fab-(scFv)2 (“tribody”) heterodimers represent up to 90% of all secreted Ab fragments in the mammalian expression system and possess fully functional binding moieties. Furthermore, both molecules recruit and activate T cells in a tumor cell-dependent way, whereby the trispecific derivative can exert this activity to two different tumor cells. Thus we propose the use of the disulfide-stabilized L:Fd heterodimer as an efficient platform for production of intermediate-sized BsAbs and TsAbs in mammalian expression systems. The Journal of Immunology, 2000, 165: 7050–7057.
scaffold for the generation of multifunctional Ab derivatives in mammalian cells.

Materials and Methods

Cell lines

HEK293T, a human embryonic kidney cell line transected with SV40 large T-Ag (SV40TAg1699) (15), was used for transient eukaryotic expression. TE2 cells are murine and CD3+ Th1-type cells are T cells (16). MO414 cells are CH3 mouse-derived MO4 fibrosarcoma cells transected with the human placental alkaline phosphatase (hPLAP) gene (17. 18). BALB/c-derived myeloma BCL1 expresses surface IgM (19).

Plasmids and gene assembly

Restriction enzymes, DNA modifying enzymes, and DNA polymerase were used as recommended by the manufacturers. DNA amplification was performed with Vent-DNA polymerase (New England Biolabs, Beverly, MA). E6 and 2C11 denote the genes or gene fragments of an anti-hPLAP and an anti-murine TCR-associated CD3ε-chain (anti-CD3) mAb, respectively. Expression plasmids were constructed in pCAGGS (20). Cloning of the L chain of E6 mAb anti-hPLAP (IgG2b/κ) in the vector pSV51E6L has been described previously (21). The E6Fd fragment encodes V_{H}, CH1, and the first five amino acids EPSGP of the upper hinge region. Gene assembly was conducted by introduction of suitable restriction sites using modifying PCR primers. All PCR-derived fragments were sequence verified after cloning. Fusions with E6CH1 or E6CL include the “elbow” regions (EMKRAD and SAAKT) from the L and Fd chains, respectively, of Fab chains. The isolated coding sequence for the CL domain was fused to the signal sequence of the E6heavychain.scFv(anti-BCL1) and (G4S)3-scFv(anti-CD3)+His6 were amplified from pQE-bssFvB1–2C11 (22) and then genetically fused to the C terminus of CL or CH1 via a DVPSPGPG or (G4S)3 linker, respectively.

Production of Ab fragments

For transient expression, HEK293T cells were transected according to the Ca{sub 2+}(PO_{4})_{2} precipitation method (23). Twenty hours before transfection, HEK293T cells were seeded at 4 × 10^{6} cells/175 cm^{2}. Fourteen micrograms DNA of each expression plasmid was added to the cells for 24 h, after which the cells were covered with supplemented DMEM containing 5 mg/L bovine insulin, 5 mg/L transferrin, and 5 μg/L selenium (ITS) replacing FCS. Medium was harvested every 48 h after transfection. Gel filtration was performed on an XK 16/88 Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ) calibrated with a commercial protein A chromatography using cobalt as a ligand. The TsAb as well as His-tagged TsAb was purified from serum with immobilized metal affinity chromatography using cobalt as a ligand. The TsAb as well as hPLAP (Sigma) were further purified on phenyl Sepharose and Q-Sepharose columns (Amersham Pharmacia Biotech).

Surface plasmon resonance

Affinity analysis was performed using a BIAcore 2000 (BIAcore, Uppsala, Sweden). Fab were prepared by papain digestion and subsequent protein A chromatography (Pierce, Rockford, IL). HMG-CoA reductase was used for MO414 fibrosarcoma cells and BCL1 lymphoma cells, respectively. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). MO414 and BCL1 tumor cells were pretreated with 50 μg/ml mitomycin C at 37°C in the dark for 12 h or 90 min, respectively. After removal of mitomycin C, 5 × 10^{6} treated cells were cocultured with 1 × 10^{6} splenocytes in a round-bottom well in the presence of 0.5 μg/ml of bispecific Fab-scFv or trispecific Fab-(scFv)_{2} molecule. After 48 h, the culture was pulsed with 0.5 μCi of [3H]thymidine (1 mCi/ml). After 18 h the cells were disrupted by freeze-thawing; the DNA was spotted on a filter and washed. The incorporated radioactivity was measured by scintillation counting (Top-Count; Packard, Meriden, CT). All experiments were performed in triplicate.

51Cr release assay

Redirecting cytotoxicity from CTL responses was assayed using a standard 51Cr release assay with syngenic CTL cells that were
allogeneically primed. Briefly, $4 \times 10^6$ splenic responder cells (C3H/HeOuico) were mixed with $4 \times 10^6$ splenic stimulator cells (C57BL/6) treated with $50 \mu g/ml$ mitomycin C for 60 min at 37°C in the dark. The mixed cell population was cocultured in 2-ml cultures in supplemented RPMI 1640 in the presence of 30 U/ml of murine IL-2. These cultures were incubated at 37°C in 7% CO₂ in humidified air for 5 days.

MO4H4 cells were incubated with 150 μCi Na²¹CrO₄ (Amer- sham Pharmacia Biotech) for 90 min at 37°C and washed carefully to minimize spontaneous release. Effector cells from the mixed lymphocyte culture were harvested and washed; $2.5 \times 10^5$ cells were plated in triplicate in 96-well U-bottom plates containing $5 \times 10^4$ tumor cells (E:T ratio 50:1) and Fab-scFv BsAb (1 μg/ml) in a total volume of 200 μl. After a 4-h incubation at 37°C, $30 \mu l$ of the culture supernatant was transferred to a Lumaplate (Packard), air dried, and counted. The percentage of specific lysis was calculated as 100 × (experimental release) − (spontaneous release)/ (maximum release) − (spontaneous release). The maximum release was the value obtained from target cells incubated with 2% SDS. Spontaneous release never exceeded 14% of the maximum release.

**Results**

**CL:CH1 heterodimerization is inefficient in mammalian cells unless enlarged with variable domains**

To assess the eukaryotic secretion of homo- and heterodimers from individual domains of Ab L and Fd chains, HEK293T cells were transiently (co)transfected with pCAGGS expression vectors containing an insert the isolated CL or CH1 domain. These domains are derived from mouse Ab E6 (IgG2b,κ) (24) specific for hPLAP (25). However, no heterodimeric product could be detected, even not if for the purpose of more sensitive detection the CH1 domain was modified with an E-tag.

To assess whether the presence of either the V₄₄ or the VL domains is required for progression of these Ab derivatives through the endoplasmic reticulum, the CL and CH1 domains were coexpressed with their corresponding extended counterparts, namely, the complete Fd chain and the native L chain, respectively (i.e., CL:VHCH1 and VLCL:CH1). Also here, no secreted heterodimers, either CL:Fd or L:CH1, could be detected. Only L monomers and L:L homodimers were demonstrated in culture fluids of L gene-(co)transfected HEK293T cells. However, coexpression of CL and CH1, both enlarged with their corresponding variable domains (in fact representing L and Fd chains) generated efficient expression of L:Fd heterodimers (Fab). The Fd chain on its own was never detectable, neither as a monomer nor as a homodimer. Thus the Fd chain can only be secreted in the form of a heterodimer. This was concluded based on the observed and expected secretion of the expected heterodimers (Fig. 1, A and B). Western blot analysis of the culture supernatant of HEK cells, cotransfected as described above, showed secretion of the expected heterodimers (Fig. 1, D and E). The secreted Ab products consist predominantly of the L-scFv:Fd and L:Fd-scFv heterodimers, along with minor bands representing L-scFv or native L chain monomers and homodimers.

Similarly, coexpression of both enlarged L scFv and Fd-scFv chains in HEK293T cells yielded a dominant L-scFv:Fd-scFv heterodimer. This was concluded based on the observed and expected 100 kDa of the dominant protein band (Fig. 1, C and F). Staining of the blotted proteins with hPLAP to reveal the specificity of the Fab portion (which is only formed in the heterodimer) confirmed the position of BsAbs and TsAbs (Fig. 1, D–F). The presence of a stabilizing disulfide bridge in Fab-scFv and Fab-(scFv)₂ heterodimers was confirmed by SDS-PAGE under reducing conditions (results not shown).

The relative amount of the secreted products was estimated by densitometric scanning of immunoreactive signals on a Western blot developed with anti-murine IgG γκ serum at different sample dilutions. Up to 90% of the secreted IgG-derived proteins was in the correct heterodimeric format when either Fd-scFv(α-CD3) was heterodimerized with the native L chain or L-scFv(α-βCL1) was heterodimerized with the Fd chain or with the L-scFv fusion product. Sometimes, an excess of L chain-derived proteins was observed, which were either in a monomeric form or appeared as a disulfide-stabilized dimer. However, Fd chain derivatives always appeared as a heterodimer. Expression levels of the heterodimeric products were estimated by calibrated immunostaining to be 3 μg/ml/24 h for both the Fab-scFv and the Fab-(scFv)₂ molecules.

**Fab-scFv BISPECIFIC AND TRISPECIFIC ANTIBODIES**

**FIGURE 1.** Heterodimerization of scFv molecules by L and Fd chains. Schematic representation of a BsAb created by extension of the C terminus of the L chain (A) or the Fd chain (B), or of a TsAb by extension of both the L and Fd chains with a scFv (C). Secretion of L:Fd heterodimers by cotransfected HEK293T cells was assayed by Western blotting and immunodetection with anti-murine IgG γκ anti-serum or with hPLAP. D, L chain elongated with scFv E, Fd chain elongated with scFv F, elongation of both L and Fd chains. Darker ellipses, CL-containing fusion molecules; brighter ellipses, CH1-containing fusion molecules. Arrowheads show respective positions on the blot. M, Molecular mass markers (kDa).
We conclude that C-terminal extension of the L and Fd chains with the various scFv does not hamper heterodimerization or secretion of the L:Fd heterodimer. Thus, the heterodimerization scaffold constitutes an instrument for efficient generation of disulfide-stabilized BsAbs and TsAbs of intermediate size (75 and 100 kDa, respectively) in mammalian cells.

**Binding characteristics of Fab-scFv and Fab-(scFv)_2 molecules**

To verify the functionality of the individual binding moieties of the anti-tumor cell/anti-T cell Fab-scFv and Fab-(scFv)_2 molecules, their binding characteristics on Ag-positive cells were determined by flow cytometry and compared with the binding characteristics of the mAbs from which the respective binding moieties were derived. As shown in Fig. 2, the (anti-hPLAP × anti-CD3) Fab-scFv molecule recognizes both hPLAP-transfected MO4I4 and CD3^+ TE2 cells. Also the (anti-hPLAP × anti-CD3 × anti-BCL1) Fab-(scFv)_2 derivative combines the binding characteristics of its donor mAbs and recognizes its Ags on MO4I4, TE2, and BCL1^+ B cells. Thus both types of Ab derivatives retained the functionality and specificity of the constituting Fab and scFv components.

To verify whether the different binding moieties of the bispecific Fab-scFv and trispecific Fab-(scFv)_2 are capable of concurrent binding to their respective Ags, we immobilized the BsAb and TsAb by binding to one of its Ags, the CD3 ligand on T cells, and detected this binding by the subsequent retention of the hPLAP Ag (Fig. 3A). In the TsAb, two more axes of bispecific binding can be determined, i.e., anti-hPLAP × anti-BCL1 and anti-CD3 × anti-BCL1. These concurrent bindings were assayed by detection of the TsAb bound to hPLAP^+ MO4I4 cells or CD3^+ TE2 cells with a biotinylated BCL1 Ag (which is a mouse IgM molecule) (Fig. 3B).

All three approaches demonstrated multiple bispecific binding along the three different axes of the TsAb.

**Fab-scFv fusion molecules mediate tumor cell-dependent activation of T cells**

The capacity of the (anti-hPLAP × anti-CD3) BsAb and the (anti-BCL1 × anti-hPLAP × anti-CD3) TsAb to recruit and activate CD3^+ T cells by its anti-T cell and anti-tumor cell reactivity was examined on the basis of a tumor cell-dependent induction of T cell proliferation and T cell-mediated cytotoxicity. Due to its dual anti-tumor cell binding moieties, the TsAb was assayed in two experimental settings, one primed with the hPLAP^+ MO4I4 tumor cells, and the other primed with the BCL1 tumor cells. As shown in Fig. 4, A and C, induction of both proliferative and cytotoxic T cell activities was apparent only in the presence of tumor cells, T cells, and BsAb or TsAb, but not in the absence of either of these components. Clearly, generation of T cell reactivity was dependent on tumor cell-induced cross-linking of the monovalent α-CD3 moiety of the BsAb or TsAb resulting from the interaction of the anti-tumor cell moiety with its corresponding tumor-associated Ag. The level of T cell reactivity depended on the amount of Ab added; the optimal concentration was determined to be between 0.1 and 1 μg/ml for both the BsAb and the TsAb (Fig. 4, B and D).

From these experiments we conclude that no conformational restrictions impair the concurrent cognitive interaction of the two binding moieties of the BsAb, and especially of the three binding moieties of the TsAb, to cross-link two different cells. As a consequence, these novel types of recombinant Abs exert activities typical of BsAbs, namely, recruitment and tumor cell-dependent...
activation of effector T cells. The TsAb can do this with two different Ags.

**Stability of L:Fd heterodimerized bi- and tribodies**

To be useful in therapy settings, recombinant Abs need to be sufficiently stable, and the recombinant model should not impair the functionality of the Ab. To check the TsAb for its possible tendency to form dimers or higher aggregates, we purified a His-tagged TsAb on a metal chelating column and subsequently applied the concentrated material on a size exclusion column (Fig. 5A). More than 95% eluted as a monomer at 100 kDa. A very small fraction of the TsAb was found as a dimer, but the material eluting in the void volume did not contain any TsAb (as determined by Western blotting and immunodetection, data not shown).

To determine the fraction of purified material that is active, the TsAb was absorbed out by immunoprecipitation with target cells. The unabsorbed fraction was detected by Western blotting and quantified by comparison to a series of 2-fold dilutions of the unabsorbed sample (Fig. 5B). Capture of the TsAb by MO4I4 or BCL1 cells resulted in a 100-fold reduction of the amount of unbound TsAb. Similar results were obtained when the corresponding anti-hPLAP and anti-BCL1 mAb were immunoprecipitated. Compared with SP2/0 cells (used as a negative control), incubation with TE2 cells (CD3$^+$) did not result in significant capture of the TsAb. However, the anti-CD3 mAb 145.2C11 was not captured effectively by the TE2 cells, likely as a consequence of the low CD3 expression levels on these T cells. In conclusion, this experiment shows that the large majority of the L-scFv:Fd-scFv heterodimers are present in a correct conformation.

We also determined the stability of the BsAb and the TsAb after incubation at 37°C in PBS or in freshly prepared mouse serum (Fig. 5C). The stability was measured as the remaining bispecific activity in the Ab samples that were incubated for different time intervals. Bispecific activity was assayed with a T cell proliferation assay. Regression analysis of the data obtained predicts half lives of the BsAb and TsAb of ~30 h when incubated in serum, as compared with 80 h when incubated in PBS ($R^2 > 0.9$). These data indicate that the biddy and triboody format is compatible with therapeutic use in vivo.

Because the effect of two C-terminal fusions on the affinity of the Fab has not been documented yet, we compared the binding
affinity of the Fab moiety in the TsAb with the original Fab prepared from the E6 mAb. According to the measured surface plasma resonance parameters listed in Table I, the affinity of the TsAb is very comparable to the affinity measured for the native Fab. This indicates that the C-terminal fusions do not induce conformational changes that affect the Ag-binding site. We conclude that the bi- and tribody molecules created have a low tendency to aggregate and can be considered as stable proteins, suitable for therapeutic use.

Discussion
For therapy, large BsAbs exhibit a better pharmacokinetic behavior compared with Ab derivatives of low m.w. However, this advantage is compromised by the strongly reduced tissue penetration of large BsAbs, a feature in which small size Ab derivatives excel. Intermediate size BsAbs, larger than Fab and smaller than whole Abs, combine the high tissue penetration of small molecules with the slow whole-body clearance of large molecules, and hence may have a better format for therapeutic applications. The drawback of these recombinant BsAbs is their requirement for efficient dimerization, essential for optimal expression and ease of purification. In this respect, heterotypic CL-CH1 interaction was shown to be a promising scaffold for production of bispecific minibodies in E. coli (13).

We propose to improve the heterodimerization efficiency of the CL-CH1 domains by using a mammalian type of expression system. Mammalian cells are known to exert a more stringent quality

FIGURE 5. Stability of Fab-scFv and Fab-(scFv)2, bi- and tribody. A, The TsAb (anti-BCL1 × anti-hPLAP × anti-CD3) was purified from cell culture supernatant and analyzed on a Coomassie Brilliant Blue-stained SDS-PAGE and on a Superdex 200 XK 26/88 (Amersham Pharmacia Biotech). The column was calibrated with standard proteins of 669, 150, 43, 13, and 0.5 kDa. The material eluting in the void (Vo) mainly contained contaminating proteins and almost no TsAb. The peaks corresponding to monomeric (M) and dimeric (D) TsAb are indicated. B, Capture of the TsAb with target cells. i, Capture of the TsAb immunodetected after SDS-PAGE with anti-mouse IgG serum shows that 1% of the starting dose can still be detected. ii, The same amount of TsAb was washed three times with MO4I (hPLAP), BCL1 (BCL1), TE2 (CD3), or SP2/0 (irrelevant) cells. iii, The same experiment was performed with the parental mAbs E6 (anti-hPLAP), B1 (anti-BCL1), and 145.2c11 (anti-CD3). C, In vitro stability of the bi- and tribody: 1 μg/ml of Ab fragment was incubated at 37°C for up to 26 h in either PBS or freshly prepared mouse serum. The stability was measured as the remaining activity in a T cell proliferation assay with MO4I (hPLAP) cells, which accounts for the presence of two specificities (anti-hPLAP and anti-CD3).
control on proteins proceeding along the secretory pathway. In particular, they contain the endoplasmic chaperone BiP/GRP78 that mainly binds to the CH1 domain of the IgH (26) and much weaker to the L chain (27). In agreement with this strict control, we never observed secreted CH1 fragments or Fd chains by themselves in the culture fluids of transfected cells, whereas L chains alone were efficiently secreted. Normally, the interaction of L chains with IgHs displaces the associated BiP and thus frees the Abs for secretion. However, coexpression of CL with CH1 or Fd did not lead to secretion of a CL:CH1 or CL:Fd heterodimer. Similar, cotransfection of L chains, instead of CL, did not result in secretion of L:CH1 heterodimers. Enlargement of CH1 with V\text{H}, resulting in formation of a Fd chain, was necessary to obtain secretion of L:Fd heterodimers in cotransfected cells. These heterodimers represented over 90% of the secreted products, reflecting tight control of their secretion. These results indicate that interaction of CL with CH1 is not efficient to free the complex for secretion, and an additional VL:V\text{H} interaction is necessary to release the CH1 domain from its endoplasmic chaperone, thus leading to efficient secretion. A similar dependence on VL:V\text{H} interactions for BiP displacement was observed in a recent study of Lee and coworkers, showing that the BiP:Fd interaction could not be displaced when the VL or V\text{H} chains were mutated to prevent secretion, and an additional VL:V\text{H} interaction is necessary to release the CH1 domain from its endoplasmic chaperone, thus leading to efficient secretion.

Having established coexpression of L and Fd as the minimal scaffold for efficient heterodimerization in mammalian cells, we analyzed the applicability of L:Fd heterodimerization for generating intermediate size BsAbs and TsAbs. To avoid possible sterical hindrance when cross-linking two cells, and to allow a better reach of intermediate size BsAbs and TsAbs. The tribody model provides an easy way to construct monovalent TsAbs, which is most valuable considering the heterogeneity of most tumors. Another application can be found in increasing the avidity for the tumor antigen or for the tumor cell.

In summary, the highly efficient heterodimerization of L and Fd chains in mammalian cells constitutes an ideal platform for generating fully functional, disulfide-stabilized BsAbs and TsAbs of intermediate size by C-terminal enlargement with one or two scFv molecules. The dependence on mammalian expression renders these novel recombinant Ab derivatives suitable for production in mammalian cell factories or transgenic flock if needed as a therapeutic agent.

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References

Table I. Comparing affinities of natural Fab' and Fab-moiety in Fab-(scFv)_2 fusion

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* Apparent affinities as measured with biologic interaction analysis using SPR on a BIAcore 2000. Fab prepared from the E6 mAb and TsAb were captured with an anti-mouse \( \alpha \) Ab coupled to a CM5 chip (BIAcore, SE). R_{max}(Ab) was consistently 500 ± 50 RU. Purified hLPAP was used as an analyte in concentrations of 200, 100, 50, 25, and 12.5 nM, at a flow rate of 10 \( \mu \)l/min. Glycine-HCl 50 mM (pH 2.0) was used for elution. The \([k_d, k_a, K_d, K_a]\) values were calculated from the sensogram using BIAeval 2.0 software.


